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PROBLEMS AND METHODS OF RESEARCH IN PROTOZOÖLOGY

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PROBLEMS AND METHODS OF RESEARCH IN PROTOZOÖLOGY

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IN THE
JOHNS HOPKINS UNIVERSITY SCHOOL OF HYGIENE AND PUBLIC HEALTH

New York
THE MACMILLAN COMPANY
1930

591.69
H362p

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Set up and printed.
Published, August, 1930.

Printed in the United States of America by
J. J. LITTLE AND IVES COMPANY, NEW YORK

PREFACE

The editors of this book have attempted to bring together information of value to students and investigators in the field of Protozoölogy that is at present either widely scattered in the literature or has never before been published. In the first half of the nineteenth century it was possible for a scholar to treat in one volume a large part of the existing knowledge of protozoölogy: but specialization has entered this field as well as other fields of science and now each student has a comprehensive knowledge of only one or a few groups of protozoa or of only one or several phases of their host-parasite relations. As a result of this tendency an investigator in one field has difficulty in keeping up with the problems and methods of research in other fields. Furthermore those who wish to take up protozoölogy as a career are unable to secure from any easily accessible sources an adequate idea of problems available for study and of methods that may be employed in their study.

This volume has been organized with the purpose of aiding both the seasoned investigator and the beginning student. The editors intended at first to prepare the entire manuscript themselves but it soon became evident that the work would be much more valuable if specialists in each field were to contribute chapters on problems and methods of research in the various phases of the subject about which they were most familiar. The response to our invitation to contribute was very gratifying and we believe that we are able to present to protozoölogists a book that will be of great value not only to their own investigations but to the general subject to which we are devoting our time and energy.

The editors wish to thank the contributors to this volume for their cooperation and also the publishers for their willingness to undertake the responsibility for a book of this type which can hardly be a financial success.

Robert Hegner
Justin Andrews

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PROBLEMS AND METHODS OF RESEARCH IN PROTOZOÖLOGY

CHAPTER I

INTRODUCTION

By

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THE science of protozoölogy includes the study of both free-living and parasitic protozoa. This book is devoted primarily to protozoa that are parasites of plants and animals. Parasitic protozoa do not differ in any fundamental respect from free-living protozoa. The principal difference between these two types is that of habitat but free-living protozoa differ among themselves and parasitic protozoa also differ among themselves in habitat about as widely as parasitic forms differ from free-living species in this respect. Both types carry on activities necessary for the maintenance of the individual and for the maintenance of the race. The individual must protect itself within its environment, secure food and carry on various physiological processes. Asexual reproduction and often sexual reproduction serve to maintain the race. A comparison of the morphology, physiology and activities of free-living and parasitic protozoa (Hegner, 1926) reveals no fundamental differences between these two types of organisms.

Protozoa are studied in various types of institutions and by men interested in a number of fields. A protozoölogist is one who is primarily interested in protozoa as a phylum of animals. Protozoölogists are usually zoölogists. If they take up the study of parasitic protozoa they usually confine themselves to the morphology, life-cycle, taxonomy and activities of the protozoön, devoting very little attention to the host. Many zoölogists who spend much of their time in the study of protozoa do not consider themselves protozoölogists but biologists since they are simply using protozoa as material for the study of certain biological phenomena. Nevertheless such investigators add much to our knowledge of the protozoa and the results of their work constitute a large part of our body of knowledge of this group of animals.

Certain protozoa are of great importance to medical men, especially those interested in tropical medicine, and to veterinarians since these organisms may be pathogenic parasites of man or domesticated animals. Hence we find that diseases in man and other animals due to these protozoa are being investigated in medical and veterinary schools. In these investigations attention is directed primarily to the effects of the parasitic invasion on the host and the studies of the parasites themselves are undertaken with particular reference to their relations to the host.

A third type of investigator interested in parasitic protozoa includes public health men who are interested in the prevention and control of protozoan diseases that occur in endemic or epidemic form. The point of view of these investigators differs from that of the other two types just described since they are primarily interested in the transmission of human protozoa and in methods of protection and control. The term host-parasite relations has been employed (Hegner, 1926) to include all of the phases of protozoölogy of interest to zoölogists, medical men, veterinarians and public health workers. A brief review of the subject of host-parasite relations is presented in Chapter II.

Protozoölogy is a comparatively new science. Because of their small size protozoa were not studied until after the microscope was invented. Free-living protozoa were discovered by the great Dutch microscopist Leeuwenhoek (1632-1723). In 1676 Leeuwenhoek prepared his findings in the form of letters which he sent to the Royal Society of London. Five years after his discovery of free-living protozoa he announced the discovery in his own stools of animalcules which, as Dobell (1920) has pointed out, must have been flagellates belonging to the species *Giardia lamblia*. Most of the early work on the protozoa was carried on with free-living species but parasitic forms were described from time to time. These did not create any considerable attention, however, until certain species were shown to be pathogenic to domesticated animals or to man. The discovery of such a pathogenic protozoön has always been followed immediately by a flood of investigations. As a rule a considerable body of knowledge regarding free-living protozoa and parasitic protozoa in lower animals was built up before similar genera and species were recorded from man. For example, free-living amœbæ were well known before amœbæ were discovered in the intestine of human beings.

The study of morphology, classification and life-cycles occupied the time of most of the early investigators of the protozoa. As more became known about the group, however, protozoa were more frequently employed for the study of genetics, animal behavior, physiology, sex, ecology, evolution and other subjects and experiment was combined with observation in their investigation. A review of the literature of protozoölogy that has appeared during the past few years indicates that although large numbers of new species are being described every year the principal investigations are devoted to experimental work and much of this work is aided by statistical methods. Probably no one can predict the direction protozoölogical investigations will take in the future but the trends seem to point toward more fundamental studies in the field of host-parasite relations.

One of the most interesting of all problems in protozoölogy is that of the evolution of the parasitic habit. Certain postulates regarding this subject seem rather definite and simple, such as, for example, that parasitic protozoa evolved from free-living protozoa and that the ectoparasitic habit evolved before the endoparasitic habit. Protozoan parasites offer particularly favorable material for the study of the course of evolution since they have become differentiated from their free-living ancestors by a sort of superimposed evolution.

The literature of protozoölogy is widely scattered. Information on the parasitic species of protozoa is contained in books, monographs and articles published in journals of various sorts. Some of the books are devoted to both free-living and parasitic species and others to parasitic species only. Books on tropical medicine all contain sections on the protozoa that produce disease in man, especially in tropical countries. A large number of journals contain articles on parasitic protozoa but not one of these is devoted entirely to this subject. Those that contain a considerable number of articles and seem to be of greatest use on this account are listed together with the more important text and reference books at the beginning of the Bibliography.

CHAPTER II

HOST-PARASITE RELATIONS BETWEEN ANIMALS AND THEIR PROTOZOAN PARASITES

By

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As pointed out in Chapter I the study of protozoölogy has been and is being carried on by men with various viewpoints. An attempt to bring together the various phases of the subject of interest to zoölogists, medical men, veterinarians and public health workers has resulted in a program which we call host-parasite relations. This program presupposes a knowledge of the morphology, taxonomy and life-cycles of the protozoa concerned. It is devoted more especially to the relations between protozoa and their hosts and involves the medical as well as the biological aspects of the subject. The writer has in previous publications presented a general outline of this program (Hegner, 1926) and a more detailed presentation of the host-parasite relations between man and his intestinal protozoa (Hegner, 1927 *c*), hence it will be unnecessary to consider *in extenso* an account of this subject.

The logical point to begin a study of host-parasite relations is with the entrance of the parasite into a clean host. This involves the study of the organism outside of the body or in an intermediate host and its method of entrance into the vertebrate host. Then follow in order the subjects of the distribution and localization of the parasites within the host, data regarding primary and secondary sites of infection, types of resistance exhibited by both host and parasite, the parasite's method of attack, changes in the host as a result of this attack as indicated by symptoms, pathological lesions and immunological reactions, changes in the parasite as a result of residence in the host, adjustments between host and parasite resulting in the carrier condition, latency and relapse, modifications in the resistance of the host due to biological or chemical therapy, the means by which infective stages in the life-cycle of the parasite escape from the body, host-parasite specificity and methods of prevention and control. These subjects may now be taken up briefly in the order indicated.

Epidemiology of transmission. Intestinal protozoa usually escape from the body in the fecal material and must be able to withstand various factors in their new environment. Most of the intestinal species produce resistant cysts which remain viable for considerable periods outside of the body, whereas trophozoites that may be carried from the body of the host quickly die. In some species, such as *Trichomonas hominis,* no cyst stage occurs in the life-cycle and transmission is effected by trophozoites. Cysts appear in most cases to be infective when they escape from the host. However, the cysts of certain species may undergo further development after they are passed, for example, those of the human coccidium. The only conceivable avenue of infection for intestinal protozoa is by way of the mouth. It seems probable that infective stages in most cases gain entrance to a new host in contaminated food or drink. This contamination may be the result of unsanitary conditions or unclean habits or may be due to the distribution of the infective stages by flies and other animals. Direct transmission or transmission by contact probably occurs in such protozoa as the flagellate and amœba that live in the human mouth and are transferred during the process of kissing.

Blood-inhabiting protozoa, such as the trypanosomes and malarial organisms, are usually sucked out of the body of the infected host by an insect, leech, or other animal, within which they pass through part of their life-cycle developing into infective stages which are subsequently inoculated into their vertebrate hosts.

In rare instances, for example, in the life-cycles of the piroplasm responsible for Texas fever in cattle, the protozoa gain entrance to the eggs of the intermediate host and the young that hatch from these eggs are thus parasitized and later become infective. This so-called "hereditary" method of transmission is, however, rare.

Clinical and parasitological periods during the course of a natural infection. It is convenient to distinguish between parasitological and clinical periods during an infection. These periods are indicated in diagrammatic form in Fig. 1. The parasitological

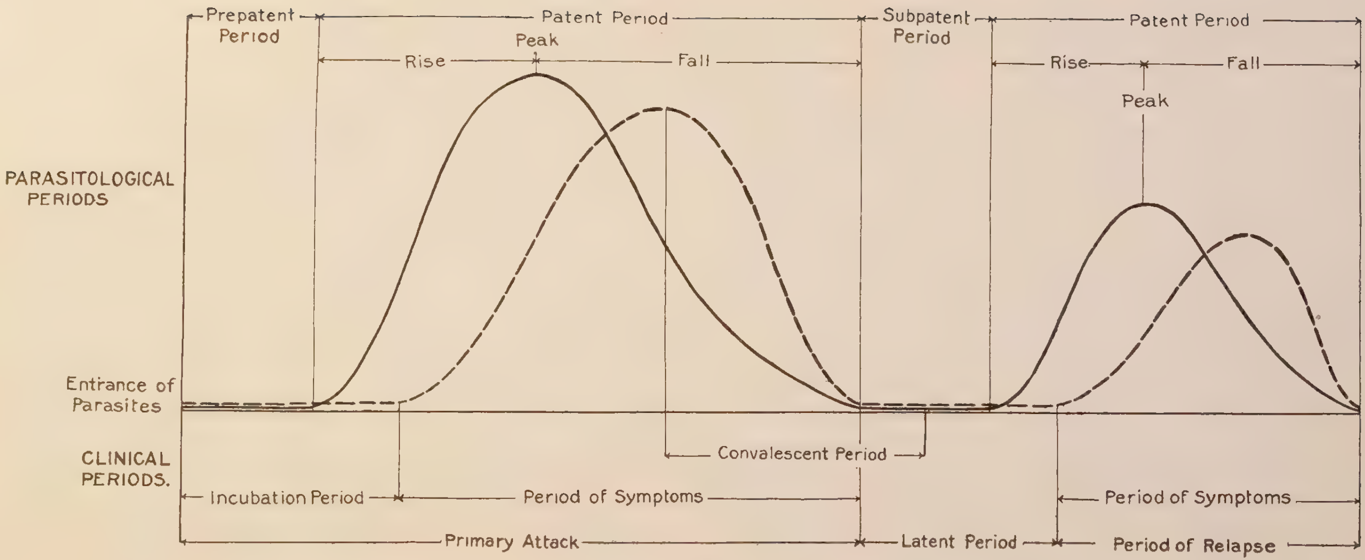

FIG. 1.—Parasitological and clinical periods, especially in protozoan infections. This illustration is intended to represent by means of curves the parasitological and clinical periods in the course of infections, especially with pathogenic protozoa. It represents in a general diagrammatic way the course of natural infections, consisting of a primary attack followed by apparent recovery of the host. This completes both the parasitological and clinical manifestations of disease in cases such as oriental sore in which one attack gives immunity. In other diseases, however, such as amœbic dysentery and malaria, the primary attack is often followed by a symptomless period when the parasites are latent. Eventually the parasite number again increases, symptoms reappear, and the host is said to suffer a relapse. The terms used are fully described in the text. (From Hegner.)

periods include a *prepatent period* which extends from the time of entrance of the parasites into the host to the time when their offspring can be recovered from the host by specified laboratory methods; a *patent period*, during which parasites can be found in the host; and a *subpatent period*, during which parasites are present but not recoverable. The subpatent period may or may not be followed by another patent period. The clinical periods include an *incubation period* which extends from the entrance of the parasites into the host to the appearance of symptoms, and a *period of symptoms* which coincides in part with the *convalescent period;* these periods complete a primary attack. A *latent period* may follow, coming to an end with the appearance of symptoms which is the beginning of a period of relapse.

Distribution of parasites within the host. Intestinal protozoa that enter the mouth in contaminated food or drink are carried through the stomach and into the intestine and thus distributed without any effort on their part but entirely by the activities of the host. Species transmitted by contact such as the flagellate and amœba of the human mouth need no further distribution. Blood-inhabiting protozoa are carried rapidly through the body by the blood stream when inoculated into the host by the intermediate host.

Primary site of infection. Each species of protozoön is more or less definitely localized in the body of the host. Some of them live in the mouth, others in the duodenum, colon, intestinal wall, the blood plasma, red blood cells, white blood cells, muscle tissue, etc.

Secondary sites of infection. Protozoa that are ordinarily localized in one region of the body may spread to other parts which become secondary sites of infection; for example, the dysentery amœba, *Endamœba histolytica,* is primarily a parasite of the large intestine where it brings about dysenteric conditions. It may, however, be transported to the liver and other organs where it is responsible for the production of abscesses.

Passive (natural) resistance of the host. Not all the stages of a parasite normal to a given host are capable of living and setting up an infection in that host and no doubt many species of foreign parasites gain entrance to hosts in which they are destroyed. The host may be said to possess a passive or natural resistance against these foreign parasites. Very little is known, however, with respect to the factors that make up this type of resistance.

Passive (natural) resistance of the parasite. Those stages in the life-cycle of protozoan parasites that are able to live and reproduce in a host possess a passive or natural resistance. Intestinal protozoa are provided in most cases with a cyst stage which successfully resists conditions in the mouth and stomach whereas, although some trophozoites of intestinal protozoa such as *Trichomonas hominis* are able to pass through the alimentary canal and reach the large intestine in a viable condition, most of them are destroyed.

The parasite's method of attack. Each species of protozoan parasite differs in its attack upon the host. Some of them appear to be harmless if present in small numbers, others attack the tissues directly and many species no doubt produce toxic substances. As a rule protozoan parasites live in harmony with their hosts. Very few of them bring about pathological conditions.

Symptomatology. The symptoms characteristic of human diseases due to parasitic protozoa are fairly well known. The protozoa bring about changes in the normal functions of the organs of the host that result in symptoms. Some of these appear at the point where injury is being done whereas others become evident in other parts of the body. The genesis of symptoms in protozoan diseases is open to experimental study but very little is known about the subject.

Pathogenesis. The pathology of many protozoan diseases is likewise fairly well known but just how the pathological lesions are produced is still to be determined in many cases. The complete pathogenesis has probably never been worked out for any protozoan disease.

Immunology. Comparatively little is known regarding immunity to protozoan infections. Different species of hosts and different individuals of the same species vary with respect to their resistance to a particular species of parasite. Acquired resistance to infection has been demonstrated in certain cases, *e.g.,* one infection with the parasite of oriental sore gives immunity to this disease.

Aggressivity. Changes in the parasite as a result of association with a host appear to take place, the aggressivity or invasive powers of the parasite becoming either less or greater. The data on this subject are meager and much work still needs to be done.

Carriers. Hosts that exhibit no visible symptoms of infection are known as carriers. Some of these hosts, known as convalescent carriers, may have shown symptoms and later recovered. Others, known as contact carriers, may never have exhibited symptoms. Most animals are probably contact carriers of one or more species of protozoan parasites since only a very small number of protozoa bring about symptoms. Carriers serve as reservoirs and are responsible for the dissemination of the parasites. There is some question as to whether certain species that sometimes produce symptoms attack the carrier host in which they live but not severely enough to bring about symptoms.

Latency. This is the term applied to conditions in which parasites are present in the host but do not bring about symptoms. Such a host is always a carrier but may not be disseminating parasites. Sometimes the term *primary latency* is used to describe the period from the entrance of the parasite into the host until symptoms appear when this period is longer than usual. *Secondary latency* may be used for the period between the primary attack and relapse.

Relapse. The reappearance of symptoms in a host that has passed through a primary attack constitutes a relapse. If the period since the primary attack is extended the reappearance of symptoms is sometimes known as a recurrence. Latent periods and relapses appear to result from reactions between the host and the parasite involving various changes in resistance and virulence about which we know very little.

Escape of parasites from the host. Many protozoa attack parts of the host from which natural channels lead to the outside through which escape is easy. For example, intestinal protozoa pass out in the feces of the host, the protozoa of the human mouth are transferred directly from one host to another during kissing and blood-inhabiting protozoa are sucked up with the blood by intermediate hosts.

Host-parasite specificity. By this term is meant the adaptability of a parasite to one or several species of hosts. When a parasite is able to live in only one species of host it is said to exhibit rigid host-parasite specificity. Host-parasite specificity is said to be weak when a parasite is able to live in several species of hosts. Various terms are used to describe these relations. For example, a host is said to be *tolerant* if it is easily parasitized by a particular species and is said to be *refractory* if it is parasitized with difficulty. A parasite is said to be a *natural parasite* if it frequently occurs in a particular host and a *foreign parasite* if it does not ordinarily parasitize a particular host species. Many interesting problems involving host-parasite specificity are available for study (Hegner, 1926).

The study of host-parasite relations as indicated in the preceding paragraphs can be directed toward one or more of the phases described. As a rule it is necessary to use material that can be obtained easily. One does not need to import parasitized animals from abroad in order to secure satisfactory types for investigation. As a matter of fact the parasites of our common laboratory and domesticated animals are very little known and problems of the greatest scientific importance can be undertaken with them. In special cases it is necessary to obtain species of parasites from foreign localities and to study them in culture or in laboratory animals but no one need hesitate to undertake investigations of host-parasite relations because of lack of material or of important problems for study.

CHAPTER III

ECTOPARASITIC PROTOZOA

By

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Many different protozoa may be found living on the external surfaces of aquatic plants and animals. Most of these use their hosts merely as a perch or conveyance; this relation may be associated with commensal habits. In some cases, the protozoa may accumulate in such numbers as to block the ready progress of their host. On the other hand, there are a few protozoa which live on the external surfaces as real parasites. Representatives of the first group will be referred to as epizoic protozoa to signify that they are not parasitic in a strict sense.

Most epizoic forms belong to the primitive flagellates, peritrichous ciliates, or SUCTORIA. Usually they are either pedunculated or sessile, though some that are placed in the group are capable of moving freely over the surface of their host. No special effort has been made to list all of the forms which have been reported and only one species is given for each genus. Some of the genera contain a large number of epizoic species. Some doubtful forms have been omitted purposely; others, such as the CHYTRIDIACEÆ, do not properly belong in this group. The SPOROZOA have been omitted because they are primarily tissue parasites.

Epizoic protozoa live in both fresh and salt water and have been found attached to practically all animals occurring in these habitats. They are especially abundant on CRUSTACEA, particularly so on the genera *Gammarus* and *Cyclops*. Only a few references are given to papers dealing with these forms. Of those given, special attention is called to the works of Kent (1880-1882), Collin (1911-1912) and Swarczewsky (1928).

In the following list some of the epizoic protozoa are given, together with their common hosts. As has been stated before, the host-parasite relation is merely one of association and usually does not involve parasitism.

Epizoic Protozoa	Hosts
Class SARCODINA:	
Amœba limax Dujardin	Tadpoles
Discella ligidi Němec	Isopods
Podarcella cancerillæ Giard	CRUSTACEA
Pontomyxa flava Topsent	Ascidians
Protoentospora ptychoderæ Sun	*Balanoglossus*
Class MASTIGOPHORA:	
Bicœca pocillum (Kent) Senn	Zoöphytes
Bodo ludibundus Senn	Tadpoles
Cephalothamnion cyclopum Stein	*Cyclops*
Codosiga candelabrum Kent	*Cyclops*
Colacium vesiculosum Ehrbg.	Copepods and Tadpoles
Elliobiopsis chattoni Caullery	Copepods
Gymnodinium pulvisculus Klebs	APPENDICULARIA
Mastigina hylæ Frenz.	Tadpoles
Oodinium fritillariæ Chatton	CRUSTACEA
Parallobiopsis coutieri Collin	CRUSTACEA
Salpingœca marina J.-Clark	Zoöphytes
Stylochrysalis parasitica Stein	*Eudorina*
Class INFUSORIA—Sub-class CILIATA:	
Order HOLOTRICHA:	
Allocaris sinensis Sollaud	CRUSTACEA
Conchopthirius anodontæ Ehrbg.	Fresh-water molluscs
Hemispeiropsis antedonis Cuenot	Echinoderms
Lionotus lamella Ehrbg.	Tadpoles
Philaster digitiformis Fab.-Dom.	Echinoderms
Pleurocoptes hydractiniæ Wallen.	HYDRO-MEDUSÆ
Uronema echini Cuenot	Echinoderms
Order PERITRICHA:	
Apiosoma piscicola Blanchard	Fish
Carchesium granulatum Kell.	*Cambarus*
Chilodochona zvennerstedti Wall.	CRUSTACEA
Cothurnia curva Stein	ENTOMOSTRACA
Epistylis anastatica Linn.	ENTOMOSTRACA
Kentrochona nebaliæ Rompel	CRUSTACEA
Lagenophrys vaginicola Stein	ENTOMOSTRACA
Licnophora auerbachi Cohn	Marine planarians
Opercularia epistyliformes Němec	*Cambarus*
Orthochona anilocræ André	Marine fish
Pebrilla paguri Giard	Hermit crab

Epizoic Protozoa	Hosts
Pyxidium cothurnoides Kent	ENTOMOSTRACA
Rhabdostyla brevipes C. & L.	Aquatic insects
Scyphidia amœbæa Grenfell	Fish
Spirochona elegans Swarcz.	*Gammarus*
Stylochona nebalina Kent	CRUSTACEA
Vorticella campanula Ehrbg.	Tadpoles
Zoothamnium parasitica Stein	CRUSTACEA
Class CILIATA—Sub-class ACINETARIA:	
Acineta notonectæ C. & L.	*Notonecta*
Acinetides varians Swarczewsky	*Gammarus*
Acinetoides graffi Plate	*Zoothamnium*
Acinetopsis rara Robin	*Sertularia*
Anophrys echini di Mauro	Echinoderms
Anoplophrya inermis Stein	Leeches
Asellicola digitata Plate	*Asellus*
Baikalodendron angustatum Swarcz.	GAMMARIDÆ
Baikalophrya acanthogammari Swarcz.	GAMMARIDÆ
Choanophrya infundibulifera Hartog	*Cyclops*
Cometodendron digitatum Swarcz.	GAMMARIDÆ
Dendrocometes astaci Stein	Crayfish
Dendrocometides priscus Swarcz.	GAMMARIDÆ
Dendrosomides paguri Collin	CRUSTACEA
Discophrya longa Swarcz.	*Gammarus*
Discosoma tenella Swarcz.	GAMMARIDÆ
Echinophrya horrida Swarcz.	*Gammarus*
Ephelota trold C. & L.	*Sertularia*
Gorgonosoma arbuscula Swarcz.	GAMMARIDÆ
Hemiophyra truncata Fraiport	*Sertularia*
Hypocoma acinetarum Collin	SUCTORIA
Lernæphrya capitata Pérez	*Cordylophora*
Ophryodendron arbietum C. & L.	HYDRAZOA
Podophrya wrzesniowski Kent	Water beetles
Rhabdophrya trimorpha Chat. & Col.	Copepods
Rhyncheta cyclopum Zenker	*Cyclops*
Stylophrya polymorpha Swarcz.	GAMMARIDÆ
Thecacineta brevistyla Swarcz.	GAMMARIDÆ
Trichophrya cordiformis Schwaikoff	*Cyclops*
Urnula epistylidis C. & L.	Pedicles of *Epistylis*

In addition to the protozoa that attach themselves to the outer surfaces of aquatic organisms, there are a few which normally live an ectoparasitic existence and are more or less dependent upon their host for sustenance. In some cases they can live for only a few hours removed from their host (*Trichodina scorpenæ*); in others they are normally free-living forms (*Prorodon teres*).

Some forms are highly pathogenic (*Hydramœba hydroxena*); others are relatively harmless and may benefit the host by keeping the surface clear of dead epithelium and detritus (*Kerona pediculus*).

There are two interesting protozoa belonging to the class SARCODINA, order PROTEOMYXA, that crawl over the surfaces of algæ and extract the cell contents through perforations which are made in the cellulose wall. One of these, *Vampyrella lateritia* (Fresenius) Cienkowski, feeds primarily on *Spirogyra;* the other, *Pseudospora volvocis* Cienkowski, feeds on *Volvox*.

A fairly exhaustive search of the literature reveals twelve genera and twenty-six species of PROTOZOA parasitic on the external surfaces of various animals; principally fishes, AMPHIBIA, flatworms and polyps.

Class SARCODINA, Order AMŒBIDA.

Amœba mucicola Chatton.

Chatton (1909) described a new species of *Amœba, A. mucicola,* found on the gills of fish. Some of the infected animals died with all signs of asphyxiation. There is a ciliate, *Trichodina labrorum,* associated with the amœbæ; it is possible that these infusoria aid the amœbæ by loosening up the epidermal cells of the host.

Description: Size when at rest 12μ to 30μ in diameter; progression by means of one or two large lobose pseudopods; found associated with *Trichodina labrorum* in the mucus on gills of fish; not proven to be an habitual parasite.

Hydramœba hydroxena (Entz) Reynolds and Looper.

Entz (1912) described an amœba which he found in great numbers, both on the outer surface and in the enteric cavity of hydras collected from pools in the vicinity of Budapest. The infected polyps showed signs of degeneration, but upon making a study of the phenomenon Entz came to the conclusion that the amœbæ were not causing the trouble. Although he observed hydra cells in the food vacuoles of the amœbæ, he considered that their principal food consisted of ciliates found in the enteron of *Hydra*. He attempted to transfer the infection to other *Hydra* but these efforts were not very successful.

Wermel (1925) carefully studied the cytology of this form. In general his findings regarding the morphology and biology of the amœbæ agree very closely with those of Entz. He states that in the neighborhood of Moscow about ninety per cent of all the hydras are infected.

Reynolds and Looper (1928) conducted a series of experiments designed to show whether or not the amœbæ were pathogenic to their host. They concluded that the amœbæ fed primarily on their host's cells. Four amœbæ with their progeny may cause death in

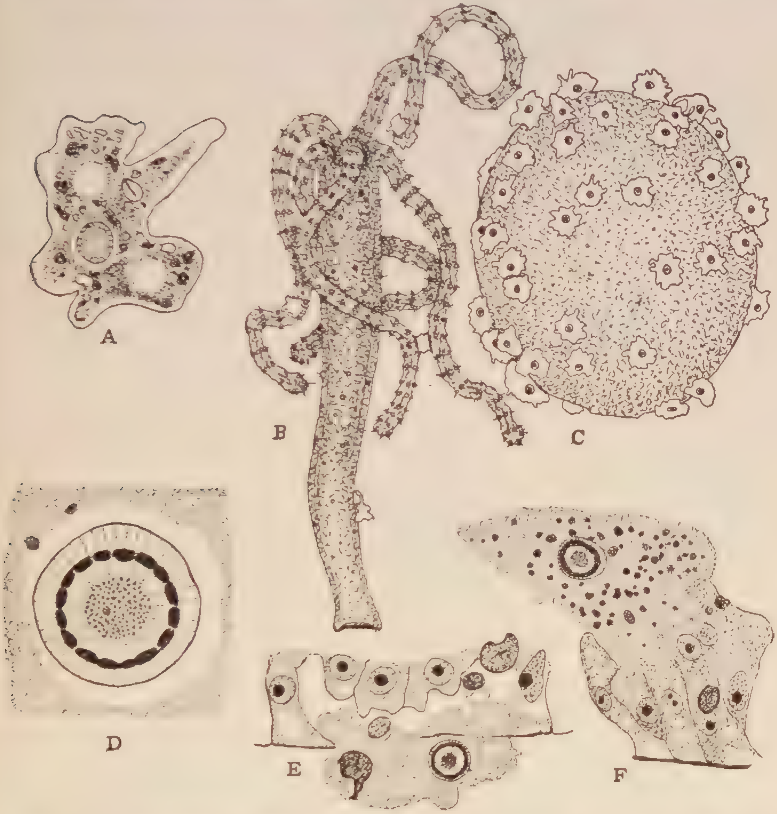

FIG. 2.—*Hydramœba hydroxena.* A, Living amœba containing two contractile vacuoles, nucleus and many food particles. B, *Hydra* during early stage of infection showing four amœbæ on tentacles and one on body. C, *Hydra* during late stage of infection, note lack of tentacles and that the amœbæ practically cover the surface. D, generalized diagram of optical section through nucleus. E, abrasion of *Hydra's* ectodermal surface by an amœba. F, amœba ingesting endodermal cells of *Hydra.* A × about 150; B × 21; C × 42; D × about 800: E and F × 210. (A after Entz; all others after Reynolds and Looper.)

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three or four days. The amœbæ may be found either on the external surface or in the enteron, but they are primarily ectoparasites.

Furthermore these authors found certain differences in the structure of the nucleus from the condition described by Entz. The question as to the identity of the two species arose, but the evidence at hand was considered inadequate to justify the erection of a new species; but a new genus was created to contain this species. Since the publication of this paper by Reynolds and Looper the author has exchanged letters and prepared slides with

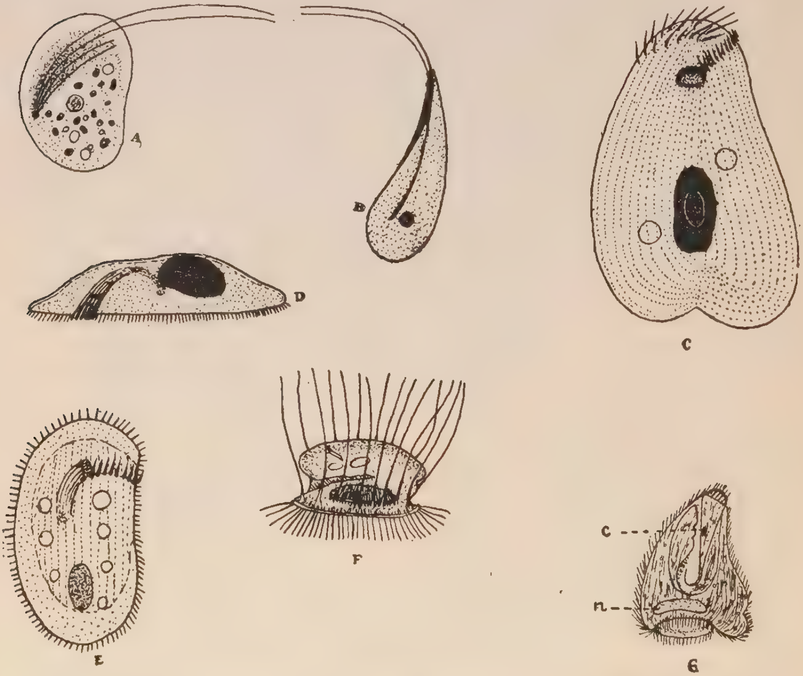

FIG. 3.—*Costia necatrix.* A, side view showing two long and two short flagella. B, as seen from edge. × 1100. (From Pascher and Lemmermann after Lemmermann.) C, *Chilodon cyprini,* ventral view showing nucleus, two vacuoles, etc., × *ca.* 700. (After Moroff.) D, *C. cyprini,* optical section showing pharyngeal basket, × *ca.* 580. (After Moroff.) E. *C. megalotrochæ,* showing nucleus and seven vacuoles, × 430. (After Stokes.) F. *Cyclochæta spongillæ.* Note crown of setæ exterior to cilia, × 290. (From Kent after Jackson.) G, *Trichodinopsis paradoxa,* (*m*), macronucleus; (*c*), cytostome, × 145. (From Bütschli after Claparède and Lachmann.)

Dr. Entz, with the result that we are in complete agreement as to the identity of the two species. It is interesting to note, however, that this protozoön has been reported from only five places: viz., Budapest (Entz), Moscow (Wermel), Baltimore (Hegner and Taliaferro), Ann Arbor (LaRue, verbally), and Charlottesville (Reynolds and Looper). In each of these places they have been found in great numbers during the autumn months. It seems that the American forms are more pathogenic than those found in Europe. Some unpublished data indicate that the concentration of hydrogen ions influences the pathogenicity of the amœbæ.

Description: Range in length from 60μ–380μ; found on external surface and in enteron of *Hydra;* die in from four to ten days if removed from host; usually cause death of host in about seven days.

Class MASTIGOPHORA, Order PROTOMONADINA.

Costia necatrix (Henneguy) Leclerque.

This flagellate was described by Henneguy (1883) under the name *Bodo necator.* It was the first ectoparasitic flagellate to be described, and was found in great numbers on the skin of young trout. The organism possesses four flagella, a long and a short pair. All of these are used in locomotion, but when attached to a fish it is anchored to the epithelium by means of the long pair, while the short pair are employed to draw dead epithelial cells into the cytostome. Since its discovery it has been reported from the skin of fish by several investigators.

Description: Size, 13μ x 20μ; flattened ovoid with anterior end narrower; found on the outer surface of fresh-water fish.

Nitsche and Weltner (1894) described a flagellate which they called *Tetramitus nitschei,* from the skin of goldfish. According to these authors *T. nitschei* differs from *C. necatrix* in several respects. It seems, however, very probable that they were dealing with the same species, or at least with the same genus.

Class INFUSORIA, Order HOLOTRICHA.

(a) *Amphileptus branchiarum* Wenrich.

Wenrich (1924) described this holotrichous ciliate from the gills of tadpoles, where they could be seen rotating actively in a thin capsular membrane. Apparently the membrane was continuous with the epithelium covering the gill. It is suggested that the ciliates burrow into the tissues and then gradually separate the cuticular membrane from the cells beneath.

The infections were not very heavy, from one to one hundred and ninety being found on the gills of one side. The mouth is capable of great expansion. The protozoön has been observed to swallow a mass of cells almost as large as itself. Wenrich states: "It is evident that the animal is a true parasite, living at the expense of its host."

Description: Average size 51.5μ x 122μ; lives in membranous capsules on the gills of tadpoles; feeds on the tissues of its host.

A. claparedei Stein has been reported from the skin of tadpoles and from the pedicles of *Epistylis*. *A. carchesi* Stein lives on the pedicles of various vorticellids. Individuals of these two species will ingest a vorticellid and then encyst on the stalk to digest the meal.

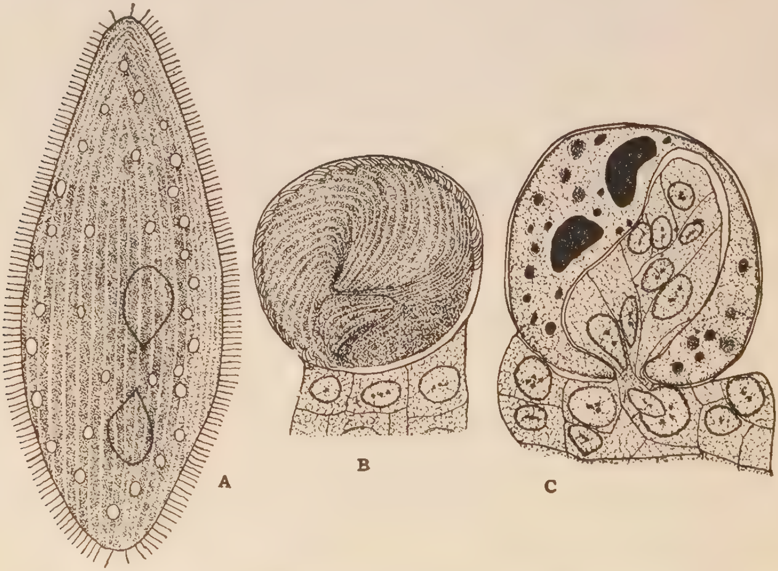

FIG. 4.—*Amphileptus branchiarum,* A, free-swimming specimen showing many vacuoles and two nuclei in outline. B, specimen in membranous capsule on gill of tadpole. C, section of specimen biting off a group of epithelial cells from the gill of tadpole, × 450. (After Wenrich.)

(b) *Chilodon cyprini* Moroff.

Moroff (1902) found a ciliate parasitic on the embryos and on the skin of young fish, especially carp, and named it *C. cyprini.* Apparently this ciliate does not live on healthy fish but attacks those sick from other causes; however, most of the infected fish died. Almost a pure culture of the ciliates could be found on the surface of some of the heavily infected fish. After the host dies the parasites leave and swim around actively in the water for a while. Soon they sink to the bottom, crawl around for a while and ultimately die.

Description: Average size 35μ x 60μ; found on embryos and on skin of sick fish; die in one to two days if removed from host.

Kiernik (1909) described another species, *C. hexastichus,* from fresh-water fish; but André (1912) maintains that this form is identical with *C. cyprini.* Goldfish were frequently attacked and killed by this form through asphyxiation. Kiernik considers that a few of these ectoparasites may be helpful to their host by destroying bacteria.

C. megalotrochæ, a parasite of the social rotifer, was described by Stokes in 1884. It glides rapidly over the surface of its host, often passing from one to another. If forced to leave its host for a long time it suffers and frequently dies.

C. longidens Němec, (1895), lives on isopods.

(c) *Enchelys parasitica* Dorier.

The genus *Enchelys* was established in 1752 by J. Hill. It contains both fresh-water and marine forms. Representatives of this genus range in size from 20μ to 200μ. Dorier (1926) describes a fatal skin disease of the rainbow trout caused by *E. parasitica.*

Description: Size 35μ to 45μ; mouth small and barely visible, located at anterior end; found on skin and gills of fish; causes extensive desquamation usually resulting in death of host.

(d) *Ichthyophthirius multifiliis* Fouquet.

From an economic viewpoint, this is the most important of the ectoparasitic protozoa. It was described by Fouquet (1876) from the skin of fresh-water fish. It is frequently the cause of disease among the young fish in hatcheries. An immature ciliate will become attached to the skin of a fish and gradually bore beneath the surface, thus forming a cutaneous pocket in which it lives. With increased growth the pocket becomes a whitish pustule which eventually ruptures, thereby liberating the enclosed parasite. This large egg-shaped ciliate sinks to the bottom and becomes surrounded by a thick cyst wall. In the encysted specimen the nucleus rapidly undergoes approximately eight successive divisions, thus giving rise to a large number of daughter nuclei. Each daughter nucleus becomes associated with a mass of cytoplasm and develops into a small ciliate which attacks a new host.

In addition to the injury caused by a loosening of the epidermis, the ciliates prepare the way for an invasion of the tissues by

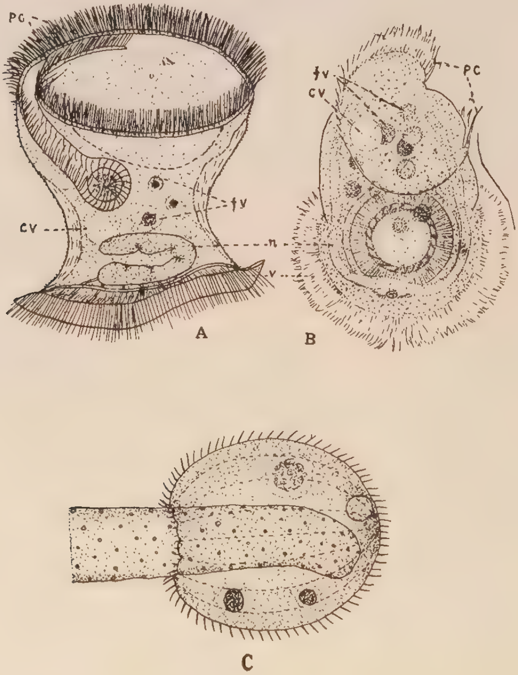

FIG. 5.—A, *Trichodina pediculus;* B, *T. steini.* (*cv*) contractile vacuole; (*fv*), food vacuoles; (*n*) macronucleus; (*pc*), peristomial cilia; (*v*), velum. × 250. (A modified from James-Clark; B after Kepner and Pickens.) C, *Prorodon teres.* The protozoön has ingested the end of a *Hydra's* tentacle; three food vacuoles and a contractile vacuole are shown. × about 50. (Modified from Reukauf.)

bacteria and fungi. In this way they may be indirectly the cause of more serious injury than they themselves produce. In some cases the ciliates accumulate on the gills of a young fish in such numbers as to cause suffocation.

Conditions favorable to the parasites are high temperature and insufficient water flow. It is not easy to kill the parasites while they are on the fish, since they withstand treatment practically as well as their hosts. Epidemics may be promptly checked, however, by a rapid circulation of fresh water in the infected ponds. Barthélémy (1926) advises that the pond be drained and exposed to sun and heat. Also the hands of workmen and the tools used should be disinfected with milk of lime or one per cent formalin.

Description: Size, adult forms 500μ–1000μ, young forms 40μ–50μ; young specimens penetrate the epithelium of soft-skinned fish and develop to maturity in epithelial pockets, then they burst out of the pustules and drop to the bottom of the ponds where they encyst and give rise by repeated divisions to about 256 small ciliates which represent the infective stage; alternates between free-living and parasitic existence.

(e) *Prorodon teres* Ehrenberg, 1883.

Ordinarily representatives of this species are free-living ciliates which feed on small animals and plants; but Reukauf (1912) records a very interesting observation in which they were found freely invading the enteric cavity of *Hydra*. He also reports that on some occasions this large gymnostomatous ciliate would enormously distend its cytostome and slip itself over the end of a tentacle, remaining there until it had digested off the end.

Description: Size 200μ x 300μ; ordinarily a free-living ciliate, but may become temporarily parasitic on *Hydra*.

Order HYPOTRICHA.

Kerona pediculus (O.F.M.) Ehrbg.

O. F. Müller was the first to give this so-called "polyp louse" a scientific name. In 1786 he gave a rather meager description of it under the name *Cyclidium pediculus*. Ehrenberg (1838) gave a very accurate description of this form, but called it *Kerona polyporum*. This has led to considerable confusion, some authors calling this ciliate *K. pediculus*, and others calling it *K. polyporum*. Apparently the latter name is a synonym for the former.

This protozoön creeps over the surface of various species of *Hydra*, feeding upon free-living protozoa and also upon the dead epithelial cells of its host. Possibly it feeds on the living cells of its host under certain conditions. Schulze (1913) observed that *Hydra* heavily infected with *K. pediculus* possessed knobbed or hypertrophied tentacles. He attributed this pathological condition to the presence of the protozoa. As this condition in *Hydra* may be induced by various other factors, Schulze's interpretation is open to question. Victor-Jones (1915) found specimens of *K. (polyporum) pediculus* both on the outer surface and in the enteron of *Hydra viridis*. He observed that when food became scarce more of the ciliates passed into the interior. He suggests that a symbiotic relationship exists between this ciliate and its host, the former keeping the surface of the latter clean of dead epithelial cells and micro-organisms which might be harmful. It is not unusual for the ciliates to leave their host temporarily and crawl around in the immediate vicinity searching for food. They are frequently found in company with *Trichodina pediculus*.

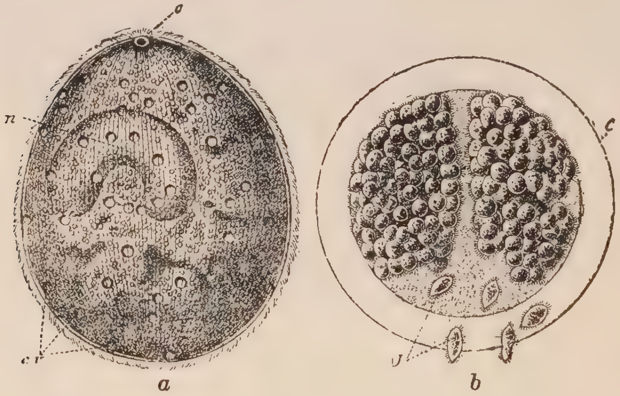

FIG. 6.—*Ichthyophthirius multifiliis*. a, vegetative stage: (*cv*), contractile vacuoles; (*n*), macronucleus; *o*, cytostome. b, cyst (*c*) from which uninucleate ciliates (*j*) are escaping. × 75. (From Hegner and Taliaferro, after Fouquet.)

Description: Size, 65μ x 130μ; body kidney-shaped, flattened dorso-ventrally; creep around on surface of hydras feeding on dead epithelial cells of host and free-living micro-organisms.

Order PERITRICHA.

(a) *Cyclochæta spongillæ* Jackson.

Jackson (1875) described a new ciliate from the outer cortical layer of the fresh-water sponge, *Spongilla fluviatilis*. It differs from the related genera, *Trichodina* and *Urceolaria* in that it possesses no anterior adoral groove and spiral of cilia and its posterior setæ form an erect fringe, the altitude of which is about twice that of the body. He established a new generic name, *Cyclochæta*, for it. It creeps about over the surface of its host very much as does *Trichodina,* apparently causing no injury.

Description: Diameter 60μ; shaped somewhat like a crown; lives on fresh-water sponges apparently as a commensal.

Wallengren (1897) describes another species, *C. domerguci* from the surface of fresh-water fish. When present in great numbers this form may cause the death of very young hosts.

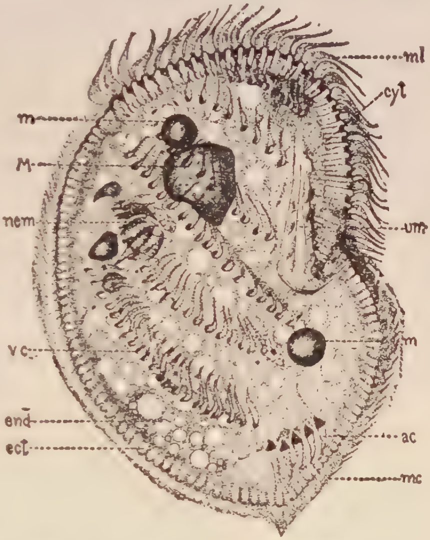

FIG. 7.—*Kerona pediculus.* Dorsal view showing *(ac)* anal cirri, *(cyt)* cytostome, *(ect)* ectoplasm. *(end)* endoplasm, *(m)* micronuclei, *(M)* macronucleus, *(mc)* marginal cirri, *(ml)* membranelles, *(nem)* ingested nematocyst, *(vc)* ventral cirri, *(um)* undulating membrane. × about 650. (From Hegner and Taliaferro, after Uhlemeyer.)

(b) *Trichodina pediculus* Ehrbg.

This is perhaps the most commonly encountered ectoparasitic protozoön. It was first fully described and named by Ehrenberg (1830). He reported it from the outer surface of *Hydra,* though it has since been reported from sponges, fresh-water medusoids, planarians, newts, tadpoles, and fish, and also as leading a free-living existence. It is more frequently found on fresh-water animals, but has also been reported from marine forms. Rosseter (1886) reported a species, which he considered similar to, but apparently not identical with *T. pediculus,* from the kidneys, testes and urinary ducts of male specimens of *Triton cristatus.* Arnold and Boulenger (1915) found specimens which showed no signs of being digested inside the circular canals of fresh-water medusoids.

This ciliate is frequently found in company with *Kerona pediculus* and has been observed to mount upon the back of the hypotrich and ride around on it. Like *K. pediculus* it is also commonly referred to as the polyp louse.

Ordinarily this protozoön merely uses its host as a means of transportation or as a place of rest; it is probable, though, that some irritation is caused to very soft-skinned hosts and thus abrasions may be made which would enable bacteria or fungi to enter. If the ciliates become very numerous they might produce death by suffocation.

Description: Diameter and length about 70μ; hour-glass-shaped; found living commensally on various aquatic animals.

Some other species of *Trichodina* that have been reported are: *T. baltica* Quennerstedt, 1869, a marine form from *Neritina fluviatilis, T. digitodiscus* Stein from hydras, *T. labrorum* Chatton, 1910, from the gills of fresh-water fish, *T. mitra* (Stein, 1854) von Siebold, from planarians, and *T. patellæ* Caullery and Mesnil, 1915, from the gills of molluscs. There is a symbiotic alga associated with this species. *T. scorpenæ* Robin, 1879, has been described from the gills of fish. According to Robin these ciliates quickly die if removed from their host. *T. steini* Claparède and Lachmann, 1858, was found on fresh-water planarians. Kepner and Pickens (1925) have pointed out that the complex adaptive structures in the basal disc of *T. steini* are but the homologues of similar structures found in *Vorticella.* According to these authors *Trichodina* has undergone a reversal of polarity. *T. synaptæ* Cuenot has been reported from echinoderms, and *T. urinicola* Fulton, 1923, from the urinary bladder of AMPHIBIA.

(c) *Trichodinopsis paradoxa* Claparède and Lachmann.

Claparède and Lachmann (1858) described this peculiar peritrichous ciliate from the respiratory surfaces and intestines of *Cyclostoma elegans.* It closely resembles *Trichodina* except for the fine cilia which cover its surface. According to Fauré-Fremiet (1909) this organism is the same as *Trichodina pediculus,* but it lives in constant symbiosis with an ectoparasitic spirillum (the fine surface cilia) and an internal bacterium.

Description: Length when extended 130μ lives on respiratory surface and in intestine of molluscs; possibly identical with *Trichodina pediculus.*

General biology and morphology: Naturally most articles deal more or less with the biology and morphology of the organism discussed; however, the works of Entz (1912), Kiernik (1909) and Wenrich (1924*a*) are especially valuable as guides to similar studies.

Comparative morphology: The works of James-Clark (1865) and Kepner and Pickens (1925) are especially helpful as guides to a comparative morphological study of the peritrichous ciliates. This phase of study has been inadequately investigated. It offers abundant material to one conversant with the general structure of protozoa.

Cytology: Wermel (1925) has made a very careful cytological study of *Hydramœba hydroxena.* This is an exceptionally good paper, but it is not readily accessible. A similar paper on the free-living protozoa might serve equally as well to guide one in a cytological study.

Nuclear division and endomyxis: Diller (1928) has described endomyxis in *Trichodina* from tadpoles. Neresheimer (1908) describes the origin of daughter nuclei from the principal nucleus in the development of *Ichthyophthirius*. The author has recently worked out the different stages in nuclear division of *Hydramœba hydroxena*. This line of investigation promises sure results and is especially recommended to the young investigator.

Life-history studies: Control measures are based primarily on a careful knowledge of life-histories. With the exception of *Ichthyophthirius multifiliis* (Fouquet, 1876) this knowledge of the ectoparasitic protozoa is inadequate. In undertaking such problems efforts should be made to culture the organisms on artificial media, as well as to make the study under natural conditions.

Symbiosis: Caullery and Mesnil (1915) have described an alga as living symbiotically with *Trichodina patellæ* and Fauré-Fremiet (1909) describes an epizoic spirillum and an internal bacterium living symbiotically with *Trichodina pediculus.* It is probable that the presence of these symbionts enables the protozoa to withstand certain conditions which otherwise would be unfavorable.

Effect of protozoa on host: It is not unusual for ectoparasitic protozoa to cause the death of their host. The pathogenic forms most commonly found belong to the holotrichous ciliates. Among these *Ichthyophthirius multifiliis* deserves first consideration. Dorier (1926) reports a very fatal skin disease of fish caused by *Enchelys parasitica.* Apparently no one has conducted carefully controlled experiments designed to show exactly what rôle the ciliates play in causing disease. Reynolds and Looper (1928) have done this for *Hydramœba hydroxena* infecting fresh-water polyps. Experiments similar to these might be employed in the study of other host-parasite relations.

Assumption of rôle as internal parasites: Probably many internal parasites have passed through a stage of external parasitism during their development of parasitic habits. Indeed some protozoa may occur as both external and internal organisms. Notable among these are *Hydramœba hydroxena* (Entz, 1912) and *Trichodinopsis paradoxa* Claparède and Lachmann (1858). Besides these, representatives of the genus *Trichodina,* which under ordinary conditions are ectoparasitic forms, have been reported from the circular canal of medusoids (Arnold and Boulenger, 1915), from the urogenital system of AMPHIBIA (Rosseter, 1886) and from the bladder of AMPHIBIA (Fulton, 1923). In light of these findings it seems possible for some of the external protozoa to be successfully transferred to internal habitats. Very little has been done along this line. The author has unsuccessfully tried to infect internally *Microstomum caudatum* with *Hydramœba hydroxena.*

Control measures: Most efforts in the direction of control measures directed against ectoparasitic protozoa involved better aëration. Barthélémy (1926) and Stiles (1893) offer suggestions concerning the control of *Ichthyophthirius.*

Host specificity: The epizoic protozoa, as well as most of those discussed under the heading ectoparasitic protozoa, usually attach themselves to several different kinds of hosts. It is seldom that host specificity does not extend to all members of a given genus, or even family, when they live under similar conditions. The following papers might be helpful to one making a study of this subject: Craig (1897), Fiebiger (1909), Reynolds and Looper (1928), and Wenrich (1924*b*). Wenrich has made a statistical study of the protozoa found on the external surface of other animals. Beginning with *Vorticella* he finds only 6.8% of the fifty-nine species associated with other animals, in *Rhabdostyla* there are 54.5% of associated species, in *Opercularia* 58.0%, in *Epistylis* 63.3%, and in *Scyphidia* 71.4%.

CHAPTER IV

RECENT CONSIDERATIONS CONCERNING THE INTESTINAL PROTOZOA OF MONKEYS

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INTRODUCTION

THE intestinal protozoa of monkeys have attracted attention primarily from two main angles of interest, the first being from a medical point of view.

Monkeys have been shown to harbor protozoa similar in morphology to those found in man and also to exhibit symptoms similar to intestinal protozoiasis of man. Macfie (1913), Eichhorn and Gallagher (1916), Fox (1923) and others have reported outbreaks of acute amœbiasis in monkeys; Kessel (1928) figures the invasion of amœbæ into the lymphatic nodules of the intestine in a monkey which showed no acute symptoms, thus illustrating chronic intestinal amœbiasis; and Castellani (1908) and Kartulis (1913) report amœbic liver abscess. These observations together with the findings of Kessel (1924*a* and 1928*d*) and Dobell (1926 and 1928) in which *Macacus* monkeys have been infected with *E. histolytica* and certain other intestinal protozoa of man indicate that monkeys may be used to advantage in the study of human intestinal protozoiasis.

The second interest has been purely theoretical and relates to the evolutionary significance of the above findings. This has been specially emphasized and popularized by Hegner (1928*b* and 1929).

EVIDENCES OF SPECIES RELATIONSHIP

When first observed, the intestinal protozoa of monkeys were given many new species names and in some instances attempts were made to draw morphological distinctions between them and their corresponding types in man. In all, six different species names have been proposed for the amœba found in monkeys that resembles *E. histolytica* of man, two for the amœba resembling *E. coli,* one for *Endolimax,* one for *Iodamœba,* two for *Trichomonas* and one for *Balantidium.* For a summary of intestinal protozoa reported from monkeys see Hegner (1928*b*) and Kessel (1928*d*).[1]

The recent tendency (see Mello, 1923; Kessel, 1926 and 1928*d;* Dobell, 1928) is to regard most of the intestinal protozoa of monkeys as being identical with the intestinal protozoa of man. Hegner (1929) has found a number of the intestinal protozoa in wild *Macacus* monkeys that have been recorded previously from captive monkeys. These claims are based on four main lines of evidence.

1. *Morphological*

With the exception of Mathis and Mercier (1917) and Fox (1923) no observers have attempted to differentiate morphologically between the intestinal amœbæ of monkeys and of man. The flagellates and *Balantidium* have not been studied as thoroughly as the amœbæ but no apparent cytological differences have been described. Hegner and Holmes (1923) and Hegner (1924) mentioned possible mensural differences between *Balantidium, Giardia* and *Chilomastix* in man and monkeys. The *Giardia* and *Chilomastix* found by Kessel (1928*d*) in monkeys, however, compare very favorably with the *Giardia* and *Chilomastix* he has found in man and he feels, if size and length to breadth ratio are to be accepted in establishing a biometric standard for species differentiation, that a greater number of cases must be observed both from man and from monkeys before such a criterion can be granted.

2. *Cultural evidence*

The growth and behavior of intestinal protozoa in artificial culture media afford a valuable means of comparing species. Evidence to date indicates that the intestinal protozoa of monkeys and of man grow with equal facility and exhibit similar behavior in the same egg-serum medium which is now in general use in culturing intestinal protozoa of man.

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[1] Through an error Dobell and Laidlaw (1926) are reported to have found *Iodamœba* in *Macacus* and to have cultured it *in vitro.*

Dobell and Laidlaw (1926) and Dobell (1928) have studied the cultural aspects of *E. histolytica*, *E. coli*, *E. nana*, *Chilomastix*, *Trichomonas* and *Enteromonas* of man and of monkeys in a very thorough and extensive investigation. They apparently have concluded that the three amœbæ from monkeys with which they have worked, are identical with their cotypes in man. The complete report of their investigation has not been published yet and they have not made a final statement concerning the species identity of intestinal flagellates.

Kessel (1926 and 1928*d*) also cultivated successfully *E. histolytica*, *E. coli*, *E. nana*, *Iodamœba*, *Chilomastix*, *Trichomonas* and *Embadomonas* from *Macacus* monkeys with which he was working in Peking and concluded that on cultural grounds these protozoa of man and of monkeys are identical.

Hegner (1928*a*) has reported the successful culture of *Trichomonas* from the vagina and intestine of *Macacus rhesus* and Knowles (1926) cultured an amœba recovered from a monkey suffering with acute amœbic dysentery.

3. *Cross-infection experiments*

Evidences of species identity of the intestinal protozoa of man and of monkeys, based on cross-infection experiments, have been presented by Walker (1913), who infected monkeys with *Balantidium coli* of man, Kessel (1924 and 1928) who infected monkeys with *E. histolytica*, *E. coli*, *E. nana*, *Iodamœba*, *Trichomonas* and *Chilomastix* of man, and by Dobell (1926 and 1928) who infected monkeys with *E. histolytica* and *E. nana* of man and also transmitted *E. nana* from *Macacus sinicus* to man. These reports lend strong evidence in favor of the species identity of the intestinal protozoa of man and of monkeys.

4. *The pathological effects of protozoa of man and of monkeys in kittens*

The kitten has been used extensively in the study of amœbiasis of man and Mello (1923), Dobell (1925 to 1928) and Kessel (1926 and 1928*d*) report acute experimental amœbiasis in kittens infected with *E. histolytica* of monkeys. In the experience of the writer the symptoms in kittens thus infected do not differ appreciably from symptoms in kittens infected with the human dysenteric amœba. Dobell (1925) mentions several points of difference but has not described them in detail as yet.

Kessel (1928*c*) also reports experimental infections of *Trichomonas* of man and of *Macacus* monkeys in kittens.

Another evidence of close species relationship between *E. histolytica* of man and of monkeys recently presented by Dobell (1928) is that he has found emetine a satisfactory therapeutic measure in amœbic infections in monkeys.

Hegner and Ratcliffe (1927) gave the name *T. macacovaginæ* to a *Trichomonas* from the vagina of a *Macacus* monkey. Hegner (1928*a*) later concluded, however, that the *Trichomonas* of the intestine and vagina of monkeys are identical so this name will become a synonym either of *T. anthropopitheci* Deschiens, 1927, of *T. vaginalis* or of *T. hominis* dependent upon whether *Trichomonas* of man and of the lower primates are concluded to be the same species and also as to whether *T. hominis* and *T. vaginalis* of man are eventually decided to be a single species. Further investigational work is necessary on this point, although Hegner's findings in the monkey lend support in favor of the hypothesis that *T. vaginalis* and *T. hominis* of man are identical.

METHODS AND PROBLEMS

Investigations with the intestinal protozoa of monkeys involve problems and methods in technique, quite similar to those encountered in studying the human intestinal protozoa, stool examinations, administration of purgatives, and cultural methods of the protozoa being the same.

Monkeys as a rule show a higher incidence of infection with intestinal protozoa than is found in man. This is undoubtedly owing to the unclean habits of the monkeys. Whether monkeys in their natural state in the tropics show a greater tendency to exhibit symptoms of acute amœbic dysentery and amœbic liver abscess than they do in captivity in more temperate regions is a problem of considerable interest, by way of comparison with human amœbiasis, since more cases of acute amœbiasis in man are found in tropical than in temperate regions.

In addition to monkeys being used as experimental animals in the solution of human amœbiasis problems they may also be of service in animal cross-infection work with lower mammals, since man ordinarily cannot cooperate in such experiments. Walker (1913) transferred *Balantidium coli* from the domestic pig to monkeys, and Brumpt (1909) infected the pig with *B. coli* of the monkey. Kessel (1928*a*) reports the successful infection of a domestic pig with *E. histolytica* of the monkey but no attempt was made to infect monkeys with the *E. histolytica*-like amœba of the pig. Numerous cross-infection experiments and many intricate problems in host-parasite relationships are awaiting solution and it seems logical to assume that monkeys may be used to advantage in investigating the same.

Hegner (1928*b* and 1929) has emphasized the evolutionary significance of the similarity between the intestinal protozoa of monkeys and of man. While this phase of the subject is of undoubted significance, the facts must not be overlooked that other domestic and laboratory animals, *e.g.*, the rat and the domestic pig, also harbor intestinal protozoa, some of which exhibit the same close relationships to the intestinal protozoa of man that are exhibited by the intestinal protozoa of monkeys. The similarity of these infections of the rat and the pig to the intestinal protozoa of man would be attributed to close association of man and these animals rather than to close evolutionary relationship, and it appears to the writer that such relations of association as well as similarity in diet between hosts should not be lost sight of in discussing the evolutionary significance of intestinal protozoa.

CHAPTER V

THE PROTOZOA OF TERMITES

By

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INTRODUCTION

Lespes in 1856 was the first to report an abundant fauna of protozoa in the intestine of a termite, *Reticulitermes lucifugus,* in France. In the latter part of the century, Leidy described the fauna in *Reticulitermes flavipes* of Eastern United States, and Grassi made known the flagellates of the two termites found in Italy, *Reticulitermes lucifugus* and *Kalotermes flavicollis.* Since that time, and especially in the last fifteen years, protozoölogists have described from these insects obtained in various parts of the world many unusually complex flagellate protozoa, together with a much smaller number of ciliates, sporozoa and amœbæ. Historical reviews of the subject have been given by Imms (1919), Koidzumi (1921), Cleveland (1923*a*) and Kirby (1926*a*) and a bibliography of the protozoa of termites up to 1926 is given in the last-named publication.

There is probably no other instance in which a protozoan infection is at the same time as abundant, diverse, constant in composition and universal in practically all individuals of the species as in this infection in termites. Except at certain stages in the life history of the insect, the flagellates in the intestine are as characteristic of the termite as any part of its anatomy. None of them are known to encyst. They are passed directly from individual to individual in some manner which is not understood. Many of the flagellates feed on particles of wood, some may ingest bacteria and small protozoa and others absorb dissolved nutriment. As shown by Cleveland (see 1926) the xylophagous flagellates appear to exist in close symbiotic partnership with their host, so that the termite is unable to survive upon the usual cellulose diet without them.

Infection by other PROTOZOA—ciliates, SPOROZOA and amœbæ—is not at all comparable in abundance to the flagellate infections. The only ciliate definitely known to occur in termites, *Nyctotherus,* has been found only in a few individuals of certain species. Some gregarines and MICROSPORIDIA are also known, and these are just as sporadic in their occurrence. Amœbæ have not been found in any termites in which the crowded faunas of flagellates occur, but an abundant infection with large forms is characteristic of at least two species of the genus *Mirotermes* in the TERMITIDÆ. Small numbers of minute amœbæ and trichomonad flagellates have been found in certain other TERMITIDÆ.

Cleveland (1925*c*) has investigated the occurrence of flagellates in the various casts of *Reticulitermes flavipes.* He found that when wood is eaten protozoa are always present, but when wood is not taken into the digestive tract these are absent. The reproductive forms lose their protozoa after they give up a diet of wood and are supported by salivary secretions from the workers. The adult soldiers, which contain protozoa, are unable to chew wood, but obtain partly digested proctodæal food from the xylophagous members of the colony. At the time of molting, all individuals of the colony lose their protozoa. Rarely a portion of the cast exoskeleton is eaten before the protozoa, discarded with the intestinal lining, are all dead, but generally the molted termites do not become reinfected if isolated. Reinfection is soon accomplished upon contact with normally faunated individuals.

DISTRIBUTION

A distributional list recently compiled by Emerson (1928) accounted for 1503 species of termites in 131 genera. With respect to intestinal PROTOZOA, these termites may be divided into two large groups, one usually with and one for the most part without infection. The former group, made up of the three most primitive of the four families, the MASTOTERMITIDÆ, KALOTERMITIDÆ and RHINOTERMITIDÆ, includes 382 species in thirty-four genera. Of these, every species so far explored has its characteristic flagellate fauna. About 110 species in forty-four genera of flagellates have been described from less than fifty of the 382 termites.

The latter group is constituted by the highest family, the TERMITIDÆ, which has 1121 species in ninety-seven genera. Many of these termites contain no protozoa, while others, which have some, nevertheless lack the large flagellate faunas of the more primitive group. The difference is correlated with the fact that the termites of the lower families feed upon wood while the TERMITIDÆ derive their sustenance from other sources than cellulose alone, such as humus, fungi and the organic matter in soil.

In the following outline of the classification of termites, a complete list of genera is given in the first three families, but in the TERMITIDÆ only those genera are listed in which some PROTOZOA have been observed. The number of known species in each genus and subgenus is quoted from Emerson (1928) and this figure is followed by one indicating the number of species which have been explored for PROTOZOA.

- Family MASTOTERMITIDÆ Silv.
  - Genus *Mastotermes* Frogg. (1) (0)
- Family KALOTERMITIDÆ Banks
- Subf. HODOTERMITINÆ Holmgren
  - Genus *Hodotermes* Hagen (21) (2)
    - Subg. *Hodotermes* s. str. (6)
      - *Microhodotermes* Sjöst. (9)
      - *Anacanthotermes* Jacobs. (6)
- Subf. TERMOPSINÆ Holmg.
  - Genus *Archotermopsis* Desn. (1) (1)
    - *Termopsis* Heer (3) (2)
    - *Hodotermopsis* Holmg. (2) (0)
- Subf. STOLOTERMITINÆ Holmg.
  - Genus *Stolotermes* Hagen (5) (0)
- Subf. KALOTERMITINÆ Holmg.
  - Genus *Porotermes* Hagen (5) (1)
  - Genus *Kalotermes* Hagen (42) (5)
    - *Epicalotermes* Silv. (1) (1)
    - *Proneotermes* Holmg. (2) (0)
    - *Neotermes* Holmg. (70) (5)
    - *Rugitermes* Holmg. (9) (1)
  - Genus *Cryptotermes* Banks
    - Subg. *Cryptotermes* s. str. (32) (4)
      - *Planocryptotermes* Light (1) (1)
  - Genus *Procryptotermes* Holmg. (6) (0)
    - *Eucryptotermes Holmg.* (1) (0)
    - *Calcaritermes* Snyder (8) (0)
    - *Glyptotermes* Frogg. (38) (3)
    - *Lobitermes* Holmg. (4) (2)
- Family RHINOTERMITIDÆ light
- Subf. PSAMMOTERMITINÆ Holmg.
  - Genus *Psammotermes* Desn. (6) (0)
- Subf. LEUCOTERMITINÆ Holmg.
  - Genus *Heterotermes* Frogg.
    - (*Leucotermes* Silv.) (21) (2)
  - Genus *Reticulitermes* Holmg. (14) (4)

Family RHINOTERMITIDÆ Light
Subf. COPTOTERMITINÆ Holmg.
  Genus *Coptotermes* Wasm. (38) (4)
    Prorhinotermes Silv. (13) (0)
Subf. TERMITOGETONINÆ Holmg.
  Genus *Termitogeton* Desn. (2) (1)
Subf. RHINOTERMITINÆ Frogg.
  Genus *Parrhinotermes* Holmg. (5) (0)
  Genus *Rhinotermes* Hagen
    Subg. *Rhinotermes* s. str. (9) (1)
      Macrorhinotermes Holmg (1) (0)
  Genus *Schedorhinotermes* *Silv.* (18) (2)
Subf. SERRITERMITINÆ Holmg.
  Genus *Serritermes* Wasm. (1) (0)

Family TERMITIDÆ Light
Genus *Cornitermes* Wasm.
  Subg. *Cornitermes* s. str. (10) (1)
Genus *Subulitermes* Holmg. (20) (2)
Genus *Amitermes* Silv.
  Subg. *Amitermes* s. str. (57) (4)
Genus *Mirotermes* Wasm.
  Subg. *Mirotermes* s. str. (44) (2)
Genus *Orthognathotermes* Holmg. (4) (1)

From a consideration of this list it is evident that exploration of termites for PROTOZOA has only begun, since of nearly 400 species which probably harbor flagellates, less than an eighth have been investigated, and the faunas of not more than five per cent

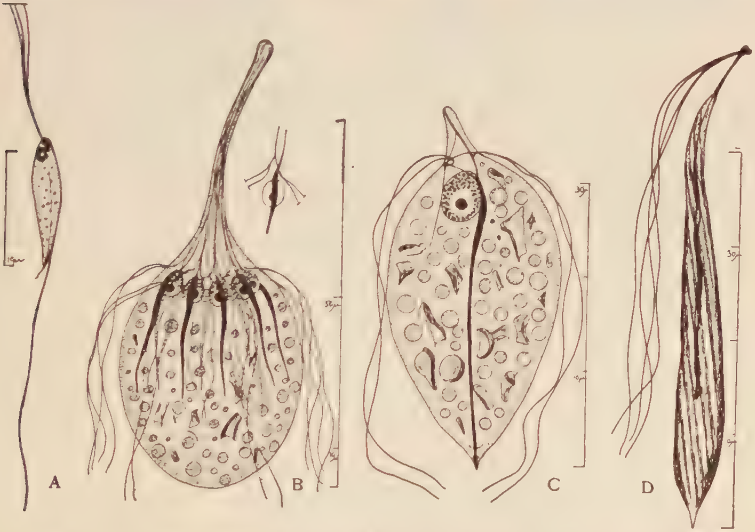

FIG. 8.—A, *Tricercomitus termopsidis* Kirby from *Termopsis angusticollis*. B, *Proboscidiella kofoidi* Kirby from *Cryptotermes dudleyi*. C, *Oxymonas granulosa* Janicki from *Neotermes connexus*. D, *Streblomastix strix* Kofoid and Swezy from *Termopsis laticeps*. B, after Kirby, 1929; A, C, D original.

are known with any degree of completeness. As the number of known termites is certainly far short of the existing number, the faunas are even more extensive than these figures indicate. Nothing is known of the PROTOZOA of *Mastotermes,* except for a statement by Hill (1922) that "ciliates (*Trichonympha*)" are present in abundance. No records have been published for *Hodotermopsis, Stolotermes, Psammotermes, Parrhinotermes, Serritermes* and several genera of the KALOTERMITINÆ. A knowledge of these would

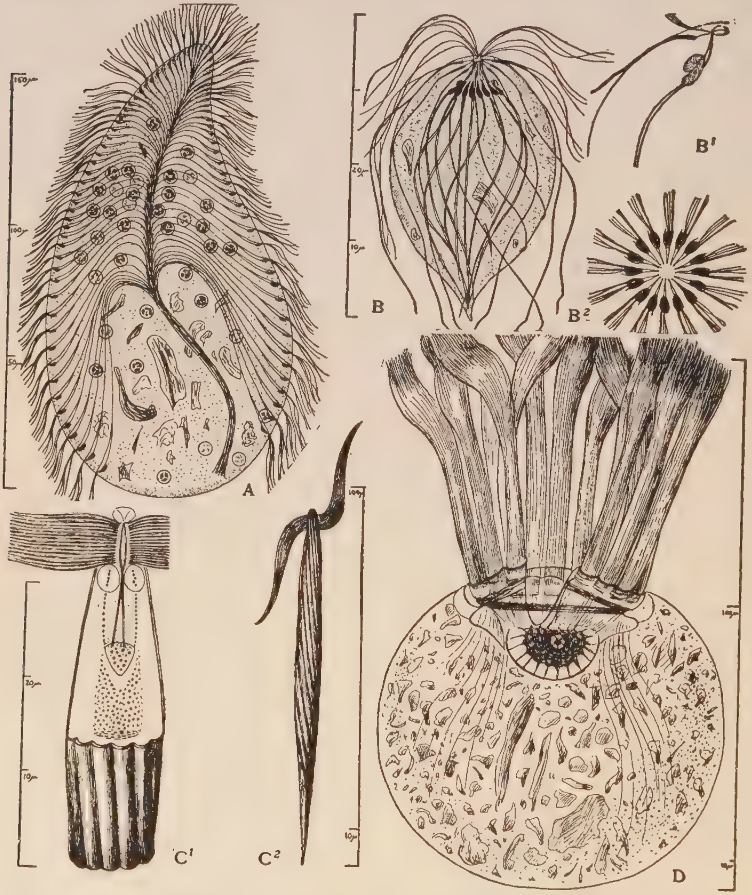

FIG. 9.—A, *Snyderella tabogæ* Kirby from *Lobitermes longicollis.* B, *Coronympha clevelandi* Kirby from *Kalotermes clevelandi;* B^1, single karyomastigont; B^2, apical view. C, *Hoplonympha natator* Light from *Neotermes simplicicornis* and D, *Kofoidia loriculata* Light from same termite. A, B after Kirby; C, D, after Light.

greatly assist in efforts to understand the distribution of flagellates in termites. In the TERMITIDÆ, the discovery of five species of unusual amœbæ in both of the only two species of *Mirotermes* s. str. which have been investigated, suggests that other interesting and important amœbæ are probably present among the forty-two other known species of that subgenus, and possibly in related termites.

CLASSIFICATION

In the following outline of classification of the flagellates in termites, no attempt is made to make the synonymy complete. The species of these genera, their hosts and the composition of the faunas of various termites have been given in Cleveland (1923), Kirby (1926*a*), Bernstein (1928) and in part in other recent papers. The figures selected are supplementary to those given by Reichenow (1928) in the fifth edition of Doflein's *Lehrbuch der Protozoenkunde*.

Order POLYMASTIGIDA Blochmann

- Family TRICHOMONADIDÆ Wenyon, 1926
  - *Tricercomitus* Kirby, 1930 (Fig. 8, A)
  - *Hexamastix* Alexieff, 1912
  - *Trichomonas* Donné, 1837
  - *Tritrichomonas* Kofoid, 1920
  - *Ditrichomonas* Cutler, 1919
  - *Trichomonopsis* Andrews, 1925
  - *Pseudotrypanosoma* Grassi, 1917
- Family DEVESCOVINIDÆ Poche, 1913
  - *Janickiella* Duboscq and Grassé, 1923
  - *Paradevescovina* Kirby, 1926
  - *Devescovina* Foa, 1905
    - Synonyms:
      - *Foaina* Janicki, 1915 (?)
      - *Caduceia* Franca, 1918
  - *Metadevescovina* Light, 1926
  - *Parajœnia* Janicki, 1911
  - *Gigantomonas* Dogiel, 1916
    - Synonym:
      - *Myxomonas* Dogiel, 1916
  - *Macrotrichomonas* Grassi, 1917
- Family STREBLOMASTIGIDÆ Kofoid and Swezy, 1919
  - *Streblomastix* Kofoid and Swezy, 1919 emend, Kidder, 1929. (Fig. 8, D)
- Family PYRSONYMPHIDÆ (Grassi, 1892) Koidzumi, 1921
  - *Pyrsonympha* Leidy, 1877
    - Synonym:
      - *Lophophora* Comes, 1910
  - *Dinenympha* Leidy, 1877
- Family OXYMONADIDÆ Kirby, 1928
  - *Oxymonas* Janicki, 1915 (Type by designation) (Fig. 8, C)
  - *Proboscidiella* Kofoid and Swezy, 1926 (Fig. 8, B)
  - *Microrhopalodina* Grassi and Foa, 1911
- Family GALONYMPHIDÆ Grassi and Foa, 1911
  - *Coronympha* Kirby, 1929 (Fig. 9, B)
  - *Stephanonympha* Janicki, 1911
  - *Diplonympha* Grassi, 1917
  - *Calonympha* (Foa, 1905) Janicki 1915
  - *Snyderella* Kirby, 1929 (Fig. 9, A)

Order HYPERMASTIGIDA Grassi and Foa, 1911

- Family LOPHOMONADIDÆ Kent, 1880
  - *Lophomonas* Stein, 1860
  - *Eulophomonas* Grassi and Foa, 1911. Genus inquirendum.
- Family KOFOIDIIDÆ Light, 1927
  - *Kofoidia* Light, 1927 (Fig. 9, D)
- Family JŒNIIDÆ Janicki, 1915
  - *Jœnia* Grassi, 1885
  - *Mesojœnia* Grassi and Foa, 1911
  - *Jœnina* Grassi, 1917
  - *Jœnopsis* Cutler, 1920
  - *Microjœnia* (Grassi, 1892) Grassi and Sandias, 1893
- Family HOPLONYMPHIDÆ Light, 1926
  - *Hoplonympha* Light, 1926 (Fig. 9, C)
- Family STAUROJŒNINIDÆ Grassi, 1917
  (STAUROJŒNIDÆ Grassi, 1917)
  - *Staurojœnina* Grassi, 1917
- Family TRICHONYMPHIDÆ (Kent, 1880) Janicki, 1915
  - *Trichonympha* Leidy, 1877
    - Synonyms:
      - *Leidyonella* Frenzel, 1891
      - *Gymnonympha* Dobell, 1910
      - *Leidyopsis* Kofoid and Swezy, 1919
- Family TRICHONYMPHIDÆ (Cont.)
  - *Pseudotrichonympha* Grassi and Foa, 1911
- Family HOLOMASTIGOTIDÆ (Janicki, 1915) Duboscq and Grassé, 1928
  - *Holomastigotes* (Grassi, 1922) Grassi and Sandias, 1893 (Fig. 10, A)
- Family SPIROTRICHONYMPHIDÆ (Grassi, 1917) Duboscq and Grassé, 1928
  - *Spirotrichonympha* Grassi and Foa, 1911 (Fig. 10, C)
    - Synonyms:
      - *Leidya* Franca, 1916
      - *Cononympha* Koidzumi, 1917
      - *Spironympha* Koidzumi, 1917
      - *Microspironympha* Koidzumi, 1921
  - *Spirotrichonymphella* Grassi, 1917
  - *Holomastigotoides* Grassi and Foa, 1911 (Fig. 10, B)
- Family CYCLONYMPHIDÆ Reichenow, 1928
  (TERATONYMPHIDÆ Koidzumi, 1921)
  - *Cyclonympha* Dogiel, 1917
    - Synonyms:
      - *Teranympha* Koidzumi, 1917
      - *Teratonympha* Koidzumi, 1921

The intestinal amœbæ from *Mirotermes* have been assigned to two genera, *Endamœba* with four and *Endolimax* with one species (Kirby, 1927). Gregarines sometimes occur in *Reticulitermes, Termopsis* and *Mirotermes.* The microsporidian *Duboscquia legeri* Perez, 1908, produces white nodules in the body cavity of *Reticulitermes lucifugus. Nyctotherus* is the only ciliate known to occur in termites, although De Mello (1921) has recorded, probably incorrectly, *Opalina, Balantidium* and a new genus *Franciella* from *Leucotermes indicola* and a species of *Coptotermes.*

PROBLEMS AND METHODS

One of the most interesting problems presented by the flagellates of termites is that of distribution in their hosts. Since they do not survive for any length of time outside of the termites and species of termites are isolated from one another, the possibilities of cross-infection are slight. It is to be expected that the presence of certain flagellates in termites will throw light on the relationship of species and the origin of genera, families and subfamilies in the ISOPTERA. Also their presence, as that of certain OPALINIDÆ in frogs and toads, will serve to indicate the relationship of hosts in different geographical regions, and the directions in which those hosts have carried their entozoic protozoa. In a survey of the uses to which host-parasite data have been and may be put to assist in the solution of problems of genetic relationship among organisms, geographical distribution and paleogeography, Metcalf (1929) states that "it seems unlikely that any other organisms will lend themselves so favorably to host-parasite studies as will the termites and their flagellates." Our present knowledge shows the value of the intestinal faunas for studies in the classification and phylogeny of the ISOPTERA, but is far too incomplete for the problem of geographical distribution. There is, however, a small amount of data which has significance for the latter question.

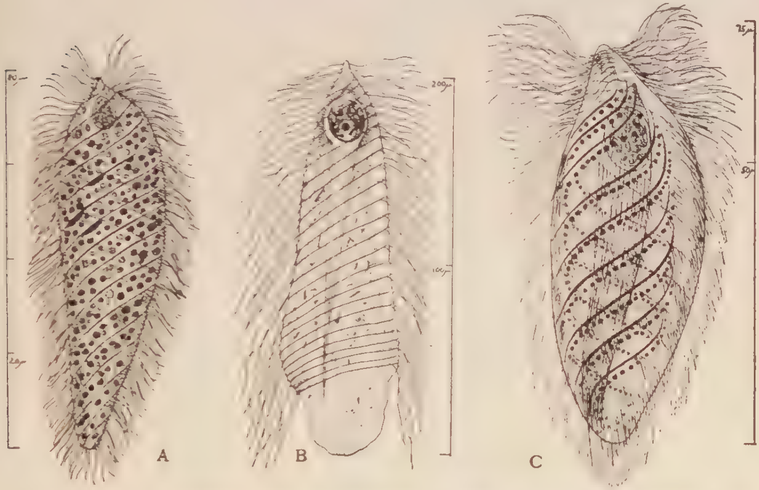

FIG. 10.—*A, Holomastigotes elongatum* Grassi from *Reticulitermes hesperus*. B, *Holomastigotoides hemigymnum* Grassi from *Leucotermes tenuis*. C, *Spirotrichonympha flagellata* Grassi from *Reticulitermes lucifugus*. A, original; B, after MacKinnon; C, after Grassi.

The following general observations on the problem of the distribution of flagellates among termites are based on records, many unpublished, from sixty-six species of termites of the three lower families, forty-four of which the writer has been able to examine.

1. In general, the faunas of more primitive termites are not of more primitive character. Highly developed hypermastigote flagellates have been found in all subfamilies of xylophagous ISOPTERA which have been investigated. Nevertheless the faunas of many KALOTERMITIDÆ are predominantly, and some entirely, polymastigote, while those of the RHINOTERMITIDÆ are chiefly, and in most cases entirely, hypermastigote.

2. *Trichomonas* and related genera and the TRICHONYMPHIDÆ are distributed through all the large groups of xylophagous termites, though they do not occur in every genus and species. Most genera of these flagellates are restricted, so far as is known, to certain families, subfamilies or genera of termites.

3. The greatest amount of flagellate specialization appears to have taken place in the subfamily KALOTERMITINÆ. Of forty well defined genera of flagellates, half are known exclusively from these termites and only ten are not known to be represented in them. At the same time it should be remembered that of the 382 species of termites belonging to the three lower families, 215 belong to the subfamily KALOTERMITINÆ. TRICHOMONADIDÆ, DEVESCOVINIDÆ, OXYMONADIDÆ and CALONYMPHIDÆ are very characteristic components of their faunas. *Trichonympha* is of frequent occurrence. Genera of the JŒNIIDÆ, *Staurojœnina* and *Spirotrichonympha* are sometimes encountered, while *Kofoidia* and *Hoplonympha* are known only from one termite, *Neotermes simplicicornis* of Arizona and desert California.

4. With a few exceptions, every species of the KALOTERMITINÆ which has been examined has a very characteristic flagellate fauna. It is usually possible to identify the termite by examination of this, or at least to distinguish between known species of a region. This provides a means of identifying nymphs, which in the absence of other casts might be confused. In most other groups of termites, the species cannot so easily be distinguished by this method. The faunas of the three species of *Termopsis* and of two species of *Porotermes* closely resemble each other. Among those of *Reticulitermes* the differences are slight and those of all other RHINOTERMITIDÆ are much alike. In the KALOTERMITINÆ, the faunas are not at all characteristic of the genera of termites as

classified at present. There is much overlapping, and resemblances are sometimes greater between the flagellates of species in separate genera than between those in the same genus. This probably indicates the need of reclassification of these termites.

5. The genus *Hodotermes* contains a fauna related on the one hand to that of the KALOTERMITINÆ and on the other to that of the RHINOTERMITIDÆ. In *Hodotermes*, there are representatives of the DEVESCOVINIDÆ, CALONYMPHIDÆ, and JŒNIIDÆ, which are well developed in the KALOTERMITINÆ. Also there are species of *Spirotrichonympha*, *Holomastigotes*, and *Holomastigotoides*, which are characteristic of the RHINOTERMITIDÆ, although *Spirotrichonympha* is found also in the KALOTERMITINÆ.

6. The RHINOTERMITIDÆ may be divided according to intestinal faunas into two markedly different groups, the first containing the genera *Heterotermes* (-*Leucotermes*). *Coptotermes, Prorhinotermes*, *Termitogeton* and *Rhinotermes* and the second only the genus *Reticulitermes*. The flagellates *Holomastigotoides, Spirotrichonympha*, and *Pseudotrichonympha* occur in the first group; *Holomastigotes, Trichonympha, Spirotrichonympha, Microjœnia* and the PYRSONYMPHIDÆ in the other.

The termites of the genera *Heterotermes* and *Reticulitermes* were formerly placed in one genus and until recently in subgenera of one genus. However, their flagellate faunas are much more distinct than are those of *Heterotermes, Coptotermes* and the other genera.

Representative material of most of the genera of flagellates in termites is obtainable within the borders of the United States. *Reticulitermes* occurs in most parts of the country. In the Pacific Coast States the large termites of the genus *Termopsis* are common, and KALOTERMITINÆ (*Kalotermes, Cryptotermes* and *Neotermes*) have been found in Georgia, Florida, Texas, Arizona and California. A species of *Prorhinotermes* which occurs in the West Indies has been found in southern Florida, and *Heterotermes aureus* has spread from Mexico into desert parts of southern California. The termites of the United States have been described by Banks and Snyder (1919).

Cleveland (1928) and Beckwith and Light (1927) have discussed the maintenance of termites in the laboratory. Filter paper may be given as food, and success in keeping the colonies depends on the proper amount of moisture. Some termites require very little; others (*Reticulitermes*) will soon die without it. *Termopsis* is easily kept indefinitely by placing a large group with wood or cellulose in a closed container. *Reticulitermes* and other termites can be kept in glass flasks or jars connected by tubes with flasks containing water. Most termites can be kept successfully in Petri dishes with filter paper as food and small pieces of cotton moistened every few days according to the amount of moisture needed.

The intestinal canal of the termite to be examined may be pulled out after grasping with forceps the extremity of the abdomen. The flagellates are for the most part localized in an expanded part of the hindgut just back of the Malpighian tubules. It is possible to force fluid from the intestines of some termites by pressure on the abdomen, without causing serious injury to the insects. Material obtained in this way, however, may not be quite representative of the fauna, in that certain attached forms will not be obtained. Since the intestinal fluid is crowded with flagellates, it is necessary to dilute it in order to make observations. Some flagellates, especially hypermastigotes, are much more sensitive to the medium used for dilution than are others. The proper concentration of the medium should be tested for the fauna of each host. In my experience a salt solution of 0.55%, or Locke's solution of two-thirds concentration, is favorable for the flagellates of *Termopsis* and *Kalotermes*. For those of *Reticulitermes,* which require a lower concentration, Powell (1928) used 0.4% salt solution, Koidzumi (1921) 0.3 to 0.4%. Egg albumen diluted with a two-thirds concentration of Locke's solution has been used to advantage.

In most media, the flagellates can be kept alive for a few hours only, and may become abnormal very quickly. Cleveland (1925) devised a fluid in which *Trichonympha* from *Termopsis* lived ten days or longer. The formula is:

$NaCl$	0.30 gm.	NaH_2PO_4	0.001 gm.
$CaCl_2$	0.02 gm.	$NaHCO_3$	0.010 gm.
KCl	0.02 gm.	H_2O	100 cc.
$MgCl_2$	0.01 gm.	Loeffler's blood serum	0.5 gm.

"This is filtered and used immediately. Instead of the serum, 5–10 grams of fresh *Termopsis* feces may be thoroughly shaken up in the above mixture, and may be used with or without filtering. If filtered, a small amount (1–2 gms.) of finely powdered lignocellulose is added." (Cleveland, 1925, p. 284.)

If a culture medium in which the flagellates will multiply can be discovered, the study of their life histories will be greatly facilitated.

For demonstration of spirochetes and other microorganisms, flagella, surface striations and other structures, dark field illumination is unsurpassed. By its use it is possible to distinguish readily between flagella and adherent microorganisms and to recognize unmistakably the number and length of flagella. Nuclei, axostyles, parabasal bodies and various cytoplasmic granules are shown distinctly. Flagella can also be demonstrated by Noland's flagellar stain or dilute picric acid or may be seen clearly enough in the larger flagellates by transmitted light.

Much confusion has resulted from the resemblance between flagella and the spirochetes which adhere to various parts of the surface of some of the flagellates. This confusion may be avoided by the use of dark field illumination or better still by removing the spirochetes from the termites by the method discovered by Cleveland (1928). This is accomplished by feeding the insects upon ground wood or filter paper soaked in a five percent aqueous solution of acid fuchsin. Under this treatment the termites thrive, at least for several months. *Kalotermes hubbardi* has been freed of all its spirochetes, including those normally attached to *Metadevescovina*, in twelve days. The protozoa seem to be little affected by this treatment, although some of them become reduced in size.

The reagents generally used in vital staining and temporary preparation of protozoa are equally valuable in the study of the flagellates of termites. Aceto-carmine kills *Trichonympha* and other flagellates in quite normal form and stains the nuclei. Acetic acid in concentration of 0.25% in 0.6% salt solution aids in bringing out nuclei, flagella and internal structures and renders many features beautifully distinct before the animal's activity ceases. Picric acid in a concentration of twenty-five per cent saturated in 0.6% salt solution has been very useful in bringing out internal structures and flagella, especially in the polymastigotes.

The cytoplasm of some of the flagellates in termites which have fed upon wood may be so crowded with wood as to make difficult the observation of structural details. These can be studied more easily when the hosts have been fed upon filter paper or starved for a short time. It is very important, however, that the flagellates be studied also from normally fed hosts, as alterations of cytoplasmic inclusions, body form and size proportions may result from cellulose feeding or starvation.

For permanent preparations smears made from the intestine of the living termite are most satisfactory. These smears can be made without dilution or with a small amount of suitable fluid. In the field, smears may be made on circular cover glasses and these stored in vials of eighty-five per cent alcohol with paper rings between them. In order to facilitate removal of the cover slips, a loose wad of cotton should be placed in the bottom of the vial and between groups of the covers. These vials should not be corked but can be stored in glass covered jars of alcohol. The intestinal canal of the termite may be pulled out and fixed entire, for later sectioning or smearing, but it is much more difficult to make satisfactory preparations from such material than from smears. Smears may also be made on slides and these stored in jars of alcohol, but in the field slides are more difficult to handle. If material is to be sent to a laboratory for study, smears should be made and entire intestines fixed in the field, in addition to the transportation of living material if this is possible. During transportation some termites may lose part or all of their fauma, though this has been true of only a small proportion of shipments.

The many methods of fixation and staining which have been used do not differ from general methods in use by protozoölogists, so that it is unnecessary to recount them all in detail. It is, however, worth while to point out those methods which have been used with most success. In fixation, best general results have been reported from the use of Schaudinn's fluid with from four to five per cent acetic acid and Bouin's fluid, either cold or heated to about 55° C. After use of these reagents, the parabasal bodies usually do not stain well. To demonstrate these, strong Flemming's without acetic, Da Fano's fixative and Champy's fluid have been used. Osmic vapor is an excellent fixative for certain purposes, especially demonstration of flagella, parabasal bodies, blepharoplasts and various granules in the cytoplasm. The smears are exposed for a short time, less than a minute, to the vapor of one per cent osmic acid, as recommended by Grassé (1926), and are then stained in iron hematoxylin.

For staining, Heidenhain's hematoxylin has been most widely used, and is unsurpassed for study of most details of cytological structure and nuclear division. In most cases, a counterstain of acid fuchsin, erythrosin, eosin, or light green is advantageous. Cutler (1919) found that Dobell's iron hæmatin gave fine results, especially for flagella and axostyles. Delafield's hematoxylin is useful chiefly for nuclear structure and for the parabasal apparatus. In most flagellates of termites with which the writer has had experience, the parabasal apparatus can be stained intensely with Delafield's hematoxylin, even after fixation by Schaudinn's fluid containing acetic acid. Alum carmine with counterstain of light green, or safranin and light green, gives fine preparations of some of the hypermastigotes.

Mallory's connective tissue stain has given some beautiful preparations of the PYRSONYMPHIDÆ, *Holomastigotes, Spirotrichonympha, Trichomonas, Macrotrichomonas* (F. C. Connell) and other flagellates. By its use the adherent flagella in the PYRSONYMPHIDÆ, flagella-bearing filaments in *Holomastigotes,* the SPIROTRICHONYMPHIDÆ and *Pseudotrichonympha,* the axostyle-like structures in the PYRSONYMPHIDÆ and *Oxymonas,* and variously coloring cytoplasmic granules and globules are brought out clearly. Parabasal bodies stain blue. Champy-Kull's method has also given brilliant results for some flagellates. Wood is stained green by this technique. The Feulgen nucleal reaction is specific for chromatin among the protoplasmic substances, but it also stains cellulose red (G. W. Kidder).

Grassé (1926) has used osmic acid to impregnate the parabasal apparatus. Following the Mann-Kopsch or Prenant-Kopsch methods, impregnation in two per cent osmic acid occupied more than a month and in *Pyrsonympha,* as in *Trichomonas batrachorum* and *Trypanosoma brucei,* about sixty days. Silver impregnation methods did not give as good results as osmic acid. For the parabasal apparatus the Smith-Dietrich technique and a method given in the text of fixation in osmic vapor, treatment with three per cent potassium bichromate, staining in Sudan III and mounting in syrup of Apathy were also successful.

It is very important to examine termites which have recently molted as well as those in which the fauna has reached its maximum abundance and become stabilized. In termites taken immediately after molting no protozoa are present. They soon become reinfected and during the first few days contain flagellates somewhat different in form and relative abundance from those which occur later on. In *Oxymonas dimorpha* from *Neotermes simplicicornis* F. C. Connell has found that a flagellated, active form occurs in recently molted nymphs and that this later becomes attached, loses its flagella and enlarges. *Tricercomitus* from *Termopsis* assumes a different form in the pale nymphs which have recently undergone ecdysis from that which occurs when the normal infection is reestablished. Investigation of the infection at various stages during the stadia of the hosts is essential for a complete understanding of the life histories of the flagellates.

Until recently, investigators have depended for mitotic figures upon random preparations. Occasionally a slide would be found on which were many stages of division, and one or a few such accidental slides provided material for an account of the process. In some instances, it was found that transferring the termites from their normal diet of wood to one of filter paper stimulated division, so that slides made a few days later contained numerous stages of the process.

Andrew and Light (1929) have investigated the problem of the occurrence of division stages and discovered methods for obtaining these in much greater abundance than earlier workers found possible. They found that after the fauna has reached its optimum number, it remains fairly constant with a very low reproductive rate and death rate, until the reduction which takes place prior to ecdysis. Following reinfection after ecdysis many division stages occur during the first few days. *Termopsis* nymphs were isolated after molting and fed the intestinal contents of normally faunated termites. On smears made from these within the first few days after reinfection mitotic figures were numerous. Division stages may be procured by taking termites which show by their coloration that they are in the third or fourth days after molting. Andrew and Light state that mitotic figures should be induced by restoration to normal conditions after partial defaunation by starvation, drying, removal of part of the fauna by pressure, heat or oxygenation. In experiments by the writer on *Termopsis* completely defaunated by oxygenation, division stages of *Trichonympha* were encountered in from a week to two weeks after placing with normally faunated nymphs.

Defaunation of termites can be accomplished by several methods discovered by Cleveland, which he has summarized in his paper of 1926. The first method he used successfully was incubation, maintaining the termites at a temperature of 36° C. for twenty-four hours, which killed all of the protozoa in *Reticulitermes flavipes*, two species of *Kalotermes* and one of *Prorhinotermes*. The method of starvation has been used chiefly for *Termopsis*, from which the large wood-feeding forms disappear in about a week when no food is provided. The smaller flagellates live for a much longer time, some of them as long as the termites. In a nymph starved for fifty-eight days, there were still a few active *Trichomonas* and *Streblomastix* and many *Tricercomitus*. The most successful method of defaunation is the use of oxygen under pressure, by which all protozoa can be removed without any apparent injury to the host. Light and Sanford (1928) have given a description of the defaunation apparatus which they used in following up Cleveland's (1925*c*) suggestions.

Some species of flagellates are more susceptible to oxygenation and deprivation of food than are others. By control of oxygenation and by starvation, or combination of the two, the removal of some species only from the fauna is possible. By various methods of treatment including feeding on different sugars, it may be possible to establish faunas of only one or a few species, and thus study those species more thoroughly than would be possible in the usual mixed infection.

The work of Light and Sanford (1927, 1928) has opened the way to an investigation of the interesting question of transfaunation of termites. There is a marked specificity in the faunas, all normally faunate individuals containing the same species of flagellates in about the same relative numbers. A consideration of the habits of termites renders it unlikely that cross-infection has taken place. Nevertheless, the question arises whether an obligatory host-parasitic specificity makes cross-infection impossible. Will the flagellates of one species of termite live in another? Light and Sanford (1927) succeeded in infecting a defaunate nymph *Porotermes* with the flagellates of *Termopsis*, and these lived in their abnormal environment for twelve days. Later (1928) they found that the flagellates of *Termopsis* could live and multiply for at least a hundred days in the intestines of nymphs of *Kalotermes hubbardi* which had been defaunated by oxygenation.

Very little is known of the physiology of cellulose digestion in termites. The work of Cleveland appears to have demonstrated that the xylophagous flagellates are essential in the process. But the action of the flagellates upon wood, the processes in the transformation of this substance in their bodies, and the manner in which it is made available to the host is not understood. That the host's food is not obtained by digestion of the protozoa is indicated by the evidence that not many of them die until shortly before the molt. Cleveland states that in *Reticulitermes flavipes,* at least, the bacteria, spirochetes and fungi living in the intestinal canal appear to play no part in cellulose digestion.

The intestinal flora of termites consists of a variety of bacteria, spirochetes, filamentous fungi and CHYTRIDIACEÆ parasitic in the protozoa. This flora has been little explored (Leidy, 1881; Hoelling, 1910; Hollande, 1922; Damon, 1926; Duboscq and Grassé, 1926, 1927; Kirby, 1927). A knowledge of it is necessary for a complete understanding of the conditions under which the PROTOZOA live. In addition to the CHRYTRIDIACEÆ parasitic in the cytoplasm and nuclei of amœbæ and many flagellates in termites, there are bacteria and other microorganisms parasitic in the flagellates.

References to citations in the text which are omitted in the Bibliography can be found in Kirby (1926), Duboscq and Grassé (1927) and Bernstein (1928).

CHAPTER VI

THE PROTOZOA OF THE ALIMENTARY TRACT OF DOMESTIC RUMINANTS AND THE HORSE

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Cattle, sheep, goats, and horses are, with the exception of the pig, our most useful domestic mammals. It would seem that our knowledge of the protozoa which may reside in the alimentary tract of these beasts would be more extensive than that of any other animal except man himself, but such is probably not the case. We hazard the guess that more work has been done on the intestinal protozoa of the rat or the frog than on those of any domestic animal. Interest in the latter, however, is developing very rapidly at the present time.

The first to make any extensive microscopical investigation of the alimentary tracts of domestic animals were Gruby and Delafond (1843), who reported in a very general way upon the protozoa which they had observed in ruminants, horses, dogs, and pigs.[1] Some of their descriptions are recognizable today; others are not. Their observations entitle them to the distinction of being called "Fathers of Veterinary Protozoölogy," for any general account of the protozoa of domestic animals must necessarily begin with them. They are credited with being the first to observe the important protozoan faunas of the rumen and reticulum of ruminants and the large intestine of the horse.

PROTOZOA OF RUMINANTS

The *rumen* (paunch) and *reticulum* (honeycomb) of the ruminant stomach are esophageal derivatives and as such contain no glands to secrete either acid or ferments. The contents consist of water and huge quantities of saliva mixed with the partially triturated food of the animal, which under ordinary farm conditions consists of either succulent or dried green plants and grain. The plants supply cellulose and lignin fibers and chlorophyll. The reaction of the fluid varies, according to the authors' experience, from slightly acid to markedly alkaline, depending upon the amount of succulent green feed the animal receives, and perhaps other factors also. This fluid serves, under normal conditions, as an ideal medium for the growth and multiplication of INFUSORIA, flagellates, amœbæ, and bacteria. It should be clearly understood that the protozoa are not to be confused with free-living forms, for they constitute a fauna more or less specific to ruminants.

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[1] It is said that Leeuwenhoek had previously seen certain protozoa from fowls.

The INFUSORIA of the stomach of domestic ruminants belong to four families. In the OPHRYOSCOLECIDÆ are the three important genera, *Ophryoscolex, Entodinium,* and *Diplodinium.* Dogiel (1927) recognizes the genus *Epidinium* Crawley, with one species and numerous varieties in domestic ruminants, as separate from *Diplodinium.* He likewise divides the genus *Diplodinium* into the subgenera *Anoplodinium, Eudiplodinium, Polyplastron,* and *Ostracodinium*—each with a number of species. The *International Rules of Zoological Nomenclature,* Article 9, states: "If a genus is divided into subgenera, the name of the typical subgenus must be the same as the name of the genus." Dogiel has disregarded this rule; hence a correction will be necessary here. In the second family, ISOTRICHIDÆ, are the genera *Isotricha* with two species and *Dasytricha* with one. In the family BUETSCHLIIDÆ is the genus *Buetschlia* with its three species. The only ruminant representatives of the PARAISOTRICHIDÆ, so common in the horse, are *Charon ventriculi* Jameson, 1925, and *Blepharocorys bovis* Dogiel, 1926. There is a possibility that they are synonyms. The authors have found *C. ventriculi* in cattle, but not *B. bovis.* For descriptions, keys, and figures of the OPHRYOSCOLECIDÆ, the best and most complete work to consult is the monograph by Dogiel (1927). A general account of the ISOTRICHIDÆ and BUETSCHLIIDÆ, as well as of certain of the OPHRYOSCOLECIDÆ, with references to other works, will be found in the report by Becker and Talbott (1927). J. Buisson (1923) has also published a useful work.

Dogiel's tables show that in cattle sixty-five species and varieties (forms) of OPHRYOSCOLECIDÆ are found; in sheep, thirty-two; in goats, nineteen. It is likely that most, if not all, of his species and varieties are genuine. Sometimes, however, one finds what seem to be intermediate forms suggesting that at least some species may fluctuate considerably. One of the authors (Becker) once found a specimen of *Diplodinium ecaudatum ecaudatum* with the posterior spine forked. It was the only one of this sort among hundreds of its species and variety observed in that one cow. Likewise, Becker and Talbott found *Ophryoscolex caudatus* in one of twenty-six cattle examined, the only one in which this genus was found at all. In this material we found two or three specimens with a rounded posterior end among hundreds bearing spines. The one without spines is supposed to be *O. inermis*. It may have been mere chance that one cow out of twenty-six examined had two species of this genus, one with the posterior armature of spines and the other without. It is more likely that these two forms were variations of one species. At present the senior author has a goat infected with *Epidinium hamatum* and a number of other species. Some specimens of this species have the typical small posterior spine. Others have spines ranging from extremely small up to the typical size. Those without spines are not *Epidinium ecaudatum ecaudatum* because the postanal portion of the posterior end is too wide.

These observations, and a number of others, suggest that species and varieties may fluctuate considerably. The alternative would be a still greater increase of species and varieties almost without end. It is necessary to know something about the variations within a pure line. This study might be made with cultures from single individuals either *in vitro* or *in vivo*. To date no one has succeeded in cultivating any infusorian from the ruminant stomach. It would, therefore, be necessary to infect azoic[2] animals. The method of obtaining such animals will be discussed below.

There is great need for a method of cultivating the INFUSORIA of the stomach of ruminants *in vitro*, not only for studies of a genetical nature, as discussed above, but also for the purpose of studying the chemical changes they may produce. Such changes might give some light on the rôle of these protozoa in the ruminant stomach. Becker and Talbott (1927) made numerous attempts to cultivate them, but failed. Rees (1927) has succeeded in cultivating

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[2] A term coined by Liebetanz for ruminants with no protozoan infection in their stomachs.

Balantidium coli, an intestinal ciliate of the pig, guinea pig, and man. His method has been tried on cattle ciliates in this laboratory with no success, but it is not improbable that some modification of it would be successful. If certain experiments *in vivo* mean anything, it appears that chlorophyll and perhaps cellulose materials will be essential to a successful culture medium.

Possible methods of obtaining pure line cultures of INFUSORIA *in vivo* should be considered. This means that, since all ruminants are infected with protozoa, it will be necessary to disinfect them of protozoa for the experiment. The goat is a very convenient laboratory animal to use. The method of disinfecting its rumen and reticulum, as worked out by Becker (1929), is as follows:

1. The animal is given no food for at least seventy-two hours. Water should be given *ad libitum.*

2. A small rubber stomach tube (as a horse catheter) is inserted into the rumen. This is accomplished by holding the goat's jaws apart by a short piece of wood (two inches square) with a hole bored in the middle through which the tube is run down the esophagus into the stomach. One must put his ear to the free end of the tube to listen for sounds of regular breathing. If these are heard the tube is in the lungs and must be withdrawn and inserted again.

3. A glass funnel is inserted into the free end of the tube. Then fifty cubic centimeters of two per cent copper sulphate in a pint of distilled water is transferred to the rumen through the funnel and tube. The goat can be put on his back and rolled so that the copper sulphate solution will become well mixed with the rumen contents. The animal does not yet receive any feed.

4. A second administration of the copper sulphate solution follows twenty-four hours after the first. Roll the goat on his back again.

5. Six hours later some oats, hay, or paper may be fed. Little will be eaten for a day or two. In another three days the goat will again be eating normally.

The animal should be kept isolated from other ruminants. He should not be permitted to drink out of a pail from which other ruminants have drunk. No hay or feed which has been "messed over" by other ruminants should be fed to it. With ordinary precautions the animals may be kept azoic.

A single protozoön might be isolated from infected material (from a ruminant stomach) by means of a Barber or other micropipette. Samples can be taken from the stomach of a goat or sheep at any time by means of the above-mentioned stomach tube and a stomach pump. The single protozoön could be put into a few drops of normal salt solution and washed down a stomach tube into an azoic goat. In a few cases out of many trials an infection might result.

There are a number of other problems in connection with this particularly interesting group of protozoa. Do they assist in cellulose digestion? Do they keep the rumen contents from putrefying by devouring excessive growth of bacteria and moulds? Do they synthesize proteins from amides, and then sacrifice their own bodies to their host in the acid or true stomach? This would make a ruminant a carnivorous animal! How is the infection spread from animal to animal? Could a calf be raised to maturity without becoming infected with INFUSORIA? These are a few of the problems involved, some of which are being worked upon at Ames. The stomach disinfection method developed by the authors will be an aid to solving all of these problems.

The flagellates of the ruminant stomach are discussed in Becker and Talbott's paper (1927), in which can be found references to other papers. *Trichomonas ruminantium, Callimastix frontalis, Eutrichomastix ruminantium*, and other flagellates are commonly seen. *Trichomonas ruminantium* was cultivated by Becker and Talbott (1927), but the others still await cultivation.

A comparative study of *Callimastix frontalis* and *Selenomastix ruminantium*, Woodcock and Lapage (1913), would be highly desirable.

Endamœba bovis is quite often encountered. It has not yet been cultivated. Becker and Frye (1927) have described its cysts which are uninucleate. Kessel (1928) finds that the cysts of *E. polecki* are of a similar type. Are *E. bovis* of ruminants and *E. polecki* of the pig identical? Here is a little problem in parasite specificity and host specificity.

Becker and Frye (1927) found in the feces of calves the trophozoites of *Trichomonas ruminantium*. There is a still smaller flagellate sometimes present which has not yet been identified. They found also the cysts of *Giardia bovis, Endamœba bovis, Buxtonella sulcata* (the identity of these cysts has been ascertained), and two species of *Eimeria* which were later identified as *Eimeria* *smithi* and *Eimeria ellipsoïdalis.* A number of problems are suggested.

Is the *Trichomonas* in the feces really *T. ruminantium* which was described from the rumen? If so, it means that the parasite is capable of living in both the stomach (rumen and reticulum) and large intestine (cecum and colon). The specimens from both places have three flagella.

What is the other small flagellate present? It might be an *Enteromonas, Tricercomonas,* or an *Embadomonas.* Hegner and Schumaker (1928) described *Embadomonas ovis* from sheep. This may occur also in cattle.

Giardia bovis was described by Fantham (1921) from the cysts and trophozoites which he found in the rumen and duodenum of cattle. Becker and Frye found that the measurements of the cysts and the ratio of mean length to mean width corresponded closely with those of *Giardia lamblia* of man. Measurements should be made of the trophozoites with a view to comparing this *Giardia* with the one in man, since the cow may serve as a natural reservoir. *Giardia* cysts have been found also in the feces of goats and sheep, but not at this laboratory. *Giardia* cysts from the cow might be fed to sheep and goats to see if they will grow there. Incidentally, an excellent stain for *Giardia* cysts is iron-hæmatoxylin counterstained with Lyon's blue, which stains the cyst wall beautifully.

Buxtonella sulcata, a ciliate belonging to the ISOTRICHIDÆ, was found by Jameson in the ceca of cattle, but never in other ruminants. Cross-infection should be attempted with sheep and goats. The cysts are present in the feces of approximately thirty per cent of all calves, according to our experience. In unpublished experiments it has been found that at least certain of the stomach INFUSORIA of ruminants are mutually interchangeable. It would not be surprising if other protozoa of ruminants showed similar relatively loose host specificity. This would be of great biological and economic significance if true of coccidia.

Hegner (1924) discovered a *Balantidium* in sheep. He states that it is considerably smaller than the one from the pig and man. Here is a question of specificity which can be investigated by the cross-infection method.

It appears to be quite definitely established that there are at least three species of *Eimeria* which may be found in cattle (*Cf.* Becker and Frye, 1929), *Eimeria zürni,* subspherical to spherical, is the pathogenic one *par excellence; Eimeria smithi*, the ovoid one, probably not of clinical importance; and *Eimeria ellipsoidalis,* with predominating ellipsoidal cysts, also probably not pathogenic. Sheep and goats likewise have their coccidia, each with probably several species. It seems extremely possible that some of the cattle coccidia may grow in sheep and goats, and *vice versa.* Cross-infection experiments should be made in order to determine their interrelationships, and it is extremely important that this be done. It is even possible that pig coccidia are from ruminant sources.

It is a problem by itself to determine whether *E. smithi* and *E. ellipsoidalis* are non-pathogenic. If they should prove to be relatively harmless, it would be extremely interesting to know if an animal which had recovered from an infection with one of them would show increased resistance to *E. zürni,* the pathogenic species. This is not to be expected from what is known about other diseases, but the matter is worth investigating.

The coprozoic flagellates found in cultures from feces are an interesting group. *Proleptomonas fæcicola* Woodcock, 1916, has a special interest because it is so nearly related to the genus *Herpetomonas,* found in insects. It would be interesting to know if it will develop in the intestines of flies. A good morphological study of it in culture would also be a contribution. One of the authors found a *Herpetomonas*-like flagellate in the rumen of the first goat examined. The flagellate has not been found again, and nothing is known about its morphology or relationships. Perhaps someone will encounter it again.

PROTOZOA OF HORSES

It will be remembered that the rumen or paunch of the ruminant stomach is the large reservoir for plant food in which cellulose digestion takes place. The horse, another herbivorous animal, seems to require also a large reservoir for the breaking down of plant fiber. Instead of an esophageal enlargement the horse is equipped with an enormous cecum and large colon. Like the rumen, the cecum and large colon are teeming with infusorian life and bacteria. Some flagellates and amœbæ may also be present.

The INFUSORIA of the horse are not so well worked up as those of ruminants. There is need of much more descriptive work. Material can usually be obtained from a rendering works where old horses are bought and killed. The contents of the cecum and large colon can be taken to the laboratory for study in a thermos bottle or jug kept at about 37° C. Ten per cent formalin is a good fixative for killing and preserving the INFUSORIA.

The best general works on these ciliates are Fiorentini (1890), Bundle (1895), Gassovsky (1919). Strelkow (1928) published a paper on the genus *Cycloposthium* only. The junior author is now preparing a monograph on the protozoa of the cecum and colon of the horse in which a number of new species will be described.

The known species of INFUSORIA from the horse fall into four families which are listed below with their genera.

Family	*Genera*
1. BUETSCHLIIDÆ Poche, 1913	*Didesmis*
	Bundleia (formerly *Buetschlia*)
	Blepharoprosthium
	Blepharosphæra
	Blepharoconus
	Holophryoides
2. PARAISOTRICHIDÆ Cunha, 1916	*Paraisotricha*
	Blepharocorys
	Blepharozoum
	Prorodonopsis
	Blepharocodon
	Paraisotrichopsis
3. CYCLOPOSTHIDÆ Poche, 1913	*Cycloposthium*
	Tripalmaria
	Tetratoxum
	Cochliatoxum
	Ditoxum
	[3] *Triadinium*
	[3] *Spirodinium*
4. PODOPHRYIDÆ Collin, 1911	[3] *Allantosoma*

From his title it appears that da Cunha (1917) has found a *Balantidium* in the horse. The authors have been unable to procure the paper to confirm this opinion, but if it is true the problems of specificity and host specificity again arise.

In connection with the protozoa of the horse there are a number of problems which are practically identical with those relating to the protozoa of ruminants, but which will probably be found to be much more difficult to solve. This is because of the difficulty of reaching the infected parts of the alimentary canal with a rubber tube for the purpose of obtaining samples of the contents. One often finds many species of the protozoa of the horse in liquid pressed from the fresh feces, but this could probably not be used as a reliable index of the presence or absence of protozoa in the cecum or colon, though this point should be carefully ascertained.

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[3] Placed in these families at least temporarily.

Among the more important problems are these: (1) Do the INFUSORIA of the horse assist in cellulose digestion? (2) Do they assist in synthesis of proteins from the amides present in the hay and grasses eaten by the horse? (3) Do they serve the horse by keeping down excessive growth of various fungi? (4) Are they pathogenic to the horse? (Fantham, 1921, indicates that *Cycloposthium* may be so.) (5) How is the infection spread from horse to horse? The INFUSORIA may be present in the feces, as stated above. But, in the absence of cysts, how can these trophozoites pass safely through the stomach to reach the large intestine?

Hsiung (1929) has described the tentacled stage of three species of SUCTORIA found in horses. Neither he nor anyone else has seen the ciliated stages of these forms. From what we know of free-living SUCTORIA, we might expect the occurrence of such stages. Search should be made for them. They should be quite small.

The ciliates of ruminants and the horse offer unparalleled opportunities for cell problems. Sharp's (1914) masterpiece on the neuromotor apparatus of *Diplodinium* will long be remembered. One of the authors (Becker) has stained the neuromotor apparatus in sections of the ciliate which appears in Becker and Talbott (1927) as *Diplodinium bursa.* It seems to be quite similar to that of *D. ecaudatum* except that the lines are finer, although the ciliate is larger. The apparatus should be worked out in *Cycloposthium, Entodinium, Ophryoscolex* and others. Sharp gives several methods of staining it. Yabroff's (1928) modified DeFano technique should also be tried.

Giardia equi found by Fantham (1921) in the colon of the horse should be restudied according to Hegner's biometric method. Like *G. bovis,* it has never been figured. It has not been found here in twenty-six horses examined.

In this chapter we have pointed out problems which should readily lend themselves to solution, except in one case, where we have stated the difficulty. Both of us are already working on some of them; but, when we contemplate the vastness of the field, the manifold aspects of each individual major problem, and the many possible methods of approach, we feel that there is little likelihood of any duplication of effort. At the present time the protozoa of domestic animals offer enough problems to employ profitably the time and effort of any aspiring students of the protozoa who have not yet located their material.

CHAPTER VII

COPROZOIC PROTOZOA

By
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INTRODUCTION

THE coprozoic protozoa are protozoan organisms which live within discharged feces.[1] They are all free-living PROTOZOA and gain access to feces either by contamination of the excrement after passage or by the entry of encysted forms, via air, food, or water, into the alimentary canal. In the latter case, the organisms do not emerge from their cysts within the intestine but excyst when the contents have been voided. Dilution of the stool, particularly in warm weather, favors the appearance of these forms.

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[1] While the author has accepted this general definition in conformity with previous publications it seems worth while to point out certain difficulties with it. Etymologically, the definition is quite sound as the word "coprozoa" is derived from two Greek words "kopros" and "zoon," meaning "dung" and "animal" respectively. Thus any animal which lives in dung is, according to the derivation of the word, quite properly called a coprozoön. Nevertheless the tendency amongst protozoölogists, while defining the term as above, is to refer to the group those organisms which have reached their fecal habitat by traversing the intestine rather than by contamination of the stool after it has been passed. There are certain exceptions to this tendency in those cases where the forms in question have been obviously introduced with the diluting fluid, but the fact that many species of very common well-known protozoa can thrive in slightly diluted feces has been either overlooked or ignored. As is indicated in the text, it is not known how many species of free-living protozoa can live in fecal cultures but the number must surely be legion. Of this large number of protozoa which are potentially capable of the coprozoic habit, probably only a few are able to reach the feces via the intestine, so that there is a well-marked physiological difference, namely, the resistance of their cysts to intestinal enzymes, between the coprozoa which were introduced to the intestinal contents by ingestion and those which reached the intestinal discharges by contamination. Whether this distinction merits redefinition of the word "coprozoa"—limiting it, perhaps, to forms which appear in feces due to ingestion but which are not capable of entozoic propagation, and reserving such an expression as "fecal contaminants" for those forms which thrive in feces after discharges but which cannot survive passage through the intestine and, therefore, must be accidentally introduced thereafter—or whether these two

Ignorance or misapprehension of the bionomics of these protozoa has in some cases led to a confusion of them with parasitic organisms or to a description of new species alleged to be entozoic. In some instances, the contaminating organisms have been introduced with the diluent used in making the smear. In order to guard against mistakes of this nature, three things should be borne in mind: (1) stools should be passed into containers which have been freshly sterilized or whose interior has never been exposed (*e.g.*, the ice-cream containers recommended by Hegner and Taliaferro, 1924, p. 483), (2) the fluid used for diluting the stool for purposes of examination should have been recently sterilized and kept free from exposure, and (3) while it is not possible to prevent the oral ingress of cysts and the consequent appearance of coprozoic trophozoites in the feces, a coprozoic fauna may be distinguished from a parasitic one by keeping the stool for a day or two and observing the relative increase in the number of organisms which will take place if they are of the coprozoic habit, or the decrease and disappearance which will occur if they are truly entozoic.

It is not known just how many species of free-living PROTOZOA can live readily in feces, but the number must be very great. In this laboratory various species of *Vorticella, Oxytricha, Stylonychia, Pleurotricha, Chilodon, Chilomonas, Colpoda, Euglena* and the "limax" amœbæ have been repeatedly encountered in stale stool cultures. Dobell (1921) has given a list of twelve organisms, most of which have been noted frequently in human stools, as an introduction to the subject. To his account the reader is referred for illustrations, bibliography, and a more detailed description than can be offered here. The following condensed descriptions of very common forms are taken largely from Dobell and O'Connor (1921) and Hegner and Taliaferro (1924).

physiologically separate groups should exist as subdivisions of the coprozoa, the writer does not feel qualified to say. The field might well be studied with the view of introducing more precision into our understanding of the bionomics of these very interesting protozoa.

SARCODINA

Dimastigamœba gruberi (Schardinger) Alexeieff, 1912.

DESCRIPTION: *active amœba*, 7μ to 15μ in diameter when rounded up, single vesicular nucleus 3μ to 4μ in diameter with central karyosome and sparse granules of "peripheral chromatin," single contractile vacuole, several large clear pseudopodia predominantly ectoplasmic while in motion, endoplasm containing ingested bacteria; *cysts*, spherical 8μ to 12μ in diameter, translucent, free from food and contractile vacuoles, uninucleate, containing several large spherical chromatoid bodies when first formed, cyst wall double, outer wall perforated by from three to eight pores; *flagellate stage*, slightly smaller than active amœba, oval, with two equal, anterior flagella, produced by flooding culture of amœbæ with distilled water and exposing to air. Found in soil, water and feces.

Trimastigamœba philippinensis Whitmore, 1911.

DESCRIPTIONS a flagellating amœba similar to *Dimastigamœba gruberi* except that it has three instead of two flagella, described from human feces by Whitmore (1911).

Tetramitus rostratus Perty, 1852.

DESCRIPTION: *active amœba*, 14μ to 48μ long, single contractile vacuole, vesicular nucleus with central karyosome and "peripheral chromatin," usually a single lobose pseudopodium; *cyst*, spherical, 6μ to 18μ in diameter, usually uninucleate (rarely bi-, tri- or quadrinucleate), cyst wall smooth and thin; *flagellate stage*, conical with apex directed posteriorly, 14μ to 18μ in length and 7μ to 10μ in greatest width, single nucleus and contractile vacuole, four anterior flagella, cytostome, flagellate form always derived from amœbic phase.

Hartmanella hyalina (Dangeard) Alexeieff, 1912.

DESCRIPTION: *trophozoite*, 9μ to 15μ in diameter when rounded, single contractile vacuole, single vesicular nucleus with central karyosome and peripheral chromatin; *cyst*, spherical, 10μ to 14μ in diameter, uninucleate, double wall, inner wall thin and smooth, outer wall thick, wrinkled, and brownish.

Sappinia diploidea (Hartmann and Nägler) Alexeieff, 1912.

DESCRIPTION: *trophozoite*, 10μ to 30μ in diameter when rounded, pellicle thick, smooth, and hyaline (rarely wrinkled), two vesicular nuclei with large central karyosome, single contractile vacuole; *cyst*, formed by two individuals, spherical, 12μ to 18μ in diameter, walls thick and uniform, single binucleate form emerges from cyst.

Chlamydophrys stercorea Cienkowski, 1876.

DESCRIPTION: *shelled amœba*, about 20μ by 14μ, shell oval, white, porcelaneous, thin, smooth, open at pointed end, pseudopodia filose, single vesicular nucleus with spherical karyosome; *naked amœba*, somewhat smaller, resembles "limax" amœbæ; *cysts*, may be formed either from shelled or naked amœbæ, uninucleate, walls thick, irregular, and brownish.

MASTIGOPHORA

Bodo caudatus (Dujardin) Stein, 1878.

Description: *flagellate,* polymorphic, from spherical to elongate oval, from 2.5μ to 6μ in breadth to 8μ to 18μ in length, permanent cytostome, minute contractile vacuole, oval kinetoplast, two small blepharoplasts from which extend two flagella, one directed anteriorly, the other nearly twice as long and directed posteriorly, single vesicular nucleus with large central karyosome; *cyst,* thin-walled, oval, 5μ to 7μ in length, single nucleus, single kinetoplast, and remains of two flagella.

Cercomonas longicauda Dujardin, 1841.

Description: *flagellate,* amœboid, 2μ to 15μ in length, one anteriorly and one posteriorly directed flagellum adherent to body and much shorter, two blepharoplasts, a single nucleus, large central karyosome, food ingested by means of pseudopodia; *cysts,* spherical, 4μ to 7μ in length, single nucleus.

Copromonas subtilis Dobell, 1908.

Description: *flagellate,* oval, 7μ to 20μ in length, permanent cytostome with cytopharynx, single vesicular nucleus, large central karyosome, single anterior flagellum longer than body, single blepharoplast on cytopharynx, contractile vacuole discharging into reservoir; *cyst,* oval or spherical, 7μ to 8μ in diameter, thin wall, clear contents.

Helkesimastix fœcicola Woodcock and Lapage, 1915.

Description: *flagellate,* oval with anterior extremity rigid and pointed, 4μ to 6μ in length, single flagellum originating in anterior end but adherent to body and free at posterior end, single vesicular nucleus, central karyosome, minute contractile vacuole, food ingested by pseudopodia; *cyst,* spherical, 3μ to 3.5μ in diameter, uninucleate.

CULTIVATION METHODS

The coprozoic protozoa are readily cultivable. Most of them will live indefinitely in a stale stool diluted with distilled water.

The coprozoic amœbæ grow well on the following solid medium devised by Musgrave and Clegg (1904) and modified by Walker (1911).

Agar	2.50 gms.
Sodium chloride	0.05 gm.
Liebig's beef extract	0.05 gm.
Normal sodium hydroxide	2.00 cc.
Distilled water	100.00 cc.

(Sterilize in autoclave. After sterilization reaction approximately neutral.)

Feces may be streaked on this medium in Petri dishes. Growth occurs at room temperature. The dishes should be inverted to prevent the water of condensation falling upon the agar. From such cultures pure lines may be started by isolation of a single individual under the binocular.

Many coprozoic flagellates and amœbæ will do well in a weak solution of egg albumen in water, hay infusion, or in a boiled watery extract of feces.

The following media were used by Bunting (1926) in the study of *Tetramitus rostratus.*

Spinach Extract

Powdered spinach	10 gms.
Distilled water	400 cc.

Boil for thirty minutes on water bath with constant stirring; filter through cotton cloth lining a heated funnel; adjust filtrate to original volume; add 0.01% Witte's peptone and 0.01% dextrose; refilter, tube and sterilize (Arnold).

An excellent solid medium can be made from the above by the addition of 1.5% agar.

Modified Sellard's Medium

Witte's peptone	1 gm.
Dextrose	1 gm.
Distilled water	100 cc.

A solid medium may be made up by adding 1.5% agar to the Modified Sellard's fluid. These media are given fractional sterilization in an Arnold sterilizer.

Modified Wherry's (1913) Ovo-Mucoid Medium

Hen's egg white	30 cc.
Distilled water	300 cc.
Yolk (optional)	1.5 cc.

The yolk was not always included in the formula and is better omitted if the medium is used for handing drops. This medium is given fractional sterilization in the Arnold and when cool the 0.5% yolk is added and thoroughly mixed with the egg white before tubing.

PROBLEMS AND METHODS

The problem of primary importance in dealing with organisms which appear in a stool is to determine whether they are of entozoic habit or whether they are coprozoic. As indicated above, probably the best way to distinguish between the two is to let the stool remain at room temperature for several days. Vegetative forms of entozoic organisms will decrease rapidly at room temperature whereas coprozoic organisms will multiply rapidly and show marked increase in numbers.

Having determined that the suspected organisms are coprozoic the next problem which presents itself is that of identification. This can be accomplished only by careful study of the organism living and in stained preparations coupled with reference to the standard works on protozoa. Calkins *Biology of the Protozoa* (1926) contains a most helpful key for the identification of unfamiliar forms. If the organism appears to be a new species its life history should be ascertained. Most of the coprozoa so far described live readily upon any nutrient medium which will maintain the fecal bacteria with which they were associated. It is well to try to cultivate unknown amœbæ or flagellates both on solid and liquid media. The solid medium is more suitable for amœbæ whereas the liquid media are correspondingly adapted for flagellates. If the organism has both an amœbic and flagellate stage this may be demonstrated by an alternation in the use of liquid and solid media. Life history studies should always be based upon observations of a pure line or clone, that is, descendants of a single individual. Single individuals may be isolated with a micropipette or a very fine glass bristle at the end of a rod or by diluting culture medium enough so that a single drop examined on a coverslip contains a single individual. The coverslip may be dropped into a test tube of fresh culture medium, thus starting a pure line. The conditions influencing the change from the amœbic phase to the flagellate phase, if such an alternation occurs, should be noted; also the factors inducing encystment.

The evolutionary significance of the coprozoic protozoa is somewhat problematical and offers an excellent field of investigation. It is generally believed by protozoölogists that entozoic protozoa are simply free-living forms that have migrated into some other animal's body and have become thoroughly adapted to that habitat. It is easy to conceive that a protozoön normally free-living that has learned to tolerate the conditions of the intestine of an animal, particularly the warm-blooded vertebrates, is taking the first step toward the adoption of the entozoic habit. It seems very probable that *Hexamita* occurs as a coprozoön and also as a harmless commensal. The organism recently described by Cleveland (1928) is also interesting from this point of view. The flagellate in question, *Tritrichomonas fecalis* Cleveland, 1928, was isolated repeatedly from the stools of one individual. The organism could not be found or cultivated until approximately four weeks after the stool had been passed. It grew luxuriantly in diluted feces and in a serum-saline medium. It was definitely proven not to be a contaminant. The relation of this organism to the individual from which it was isolated is one of interesting speculation.

The factors which influence excystation in these forms deserves more study. The cysts are apparently resistant to intestinal enzymes as they do not hatch within the body of the animal through which they are passing yet they excyst readily in the diluted discharges at room temperature. Dobell and O'Connor (1921) have suggested the high temperature of the warm-blooded vertebrates and the absence of free oxygen as possible conditions which prevent coprozoic forms from multiplying within the intestine. Possibly these same factors are concerned with the mechanism for excystation. These factors and many similar ones might be studied profitably to explain the mode of excystation and the general bionomics of the coprozoic protozoa.

CHAPTER VIII

THE CULTIVATION OF INTESTINAL PROTOZOA

By
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Charlotte, N. C.

PROBLEMS TO BE CONSIDERED IN CULTIVATION

The first reports of cultivation of intestinal PROTOZOA, other than amœbæ, are, in the main, instances of keeping organisms alive in various forms of culture medium. The numerous early reports of the cultivation of parasitic amœbæ were based on the use of free-living amœbæ, instead of parasitic amœbæ. In attempting to cultivate protozoa on artificial media, many problems present themselves:

(a) First of all, the medium should be one which has no harmful effect on the organism. In other words, a medium in which the organism will remain alive over a long period of time—a medium in which an organism will live is not necessarily one in which it will multiply. For example, many protozoa will live for days, or even weeks, in different concentrations of salt solution, although they do not multiply in this solution.

(b) Another important consideration is the reaction or hydrogen-ion concentration of the medium.

(c) Still another consideration is the oxygen tension, which is controlled best, in many media, by the height of the column of the medium in the tube, or other container used.

(d) An important consideration, necessarily, is providing a constituent in the medium which will furnish nourishment to the organism under cultivation. The nourishing constituent of the medium should be supplied in a form available to the organism.

(e) A final consideration is the proper temperature not only for maintaining the life of the organism, but a temperature in which the organism will multiply.

The cultivation of an organism, even though by a method which has previously been found a successful one, is often attended by numerous failures. Some of the common reasons for failure are:

(a) The presence of bacteria, in the material used, which form products harmful to the growth of the protozoa present. The great variety of intestinal bacteria, and the constant change from day to day in the intestinal flora easily explains why an attempted culture of intestinal protozoa may be successful at one time, and not at another.

(b) Another common cause for failure is the presence of other protozoa which may grow more rapidly than the organism under attempted cultivation. The yeast-like organism, *Blastocystis,* has been found in the hands of the writer to be one of the most troublesome contaminants.

(c) A third reason for failure is the use of material which has been kept for too long a time after removal from its original habitat.

SUGGESTED METHODS OF APPROACHING PROBLEMS OF CULTIVATION OF ANY ORGANISM

In approaching a problem of cultivation, it is essential that provisions be made for the following:

1. A plentiful supply of the organism to be cultivated.
2. A means of preserving the organism in as fresh state as possible while attempting its cultivation, keeping it free from exposure to the air and refraining from mixing it with unsuitable media.
3. The determination of the proper concentration of the simple medium used as the base for a culture fluid.
4. The determination of the proper range of hydrogen-ion concentration in the basic medium.
5. The determination of the proper substance, or substances, for the nourishment of the organism.
6. The determination of the proper oxygen requirements of the organism. This is best controlled by the height of the column in the tube of medium used.
7. The determination of the proper oxygen tension. This is best controlled by heating the upper portion of the tube at

various temperatures, and inserting immediately a rubber stopper.

8. The determination of the most favorable temperature for growth of the organism in question.
9. The determination of the correct intervals for making transfers.

In discussing the approach to the problem of cultivating an organism, for which the method has not previously been worked out, it might be of interest to outline a plan recently used in attempting to cultivate *Opalina*. In studying this outline, the reader will note that the suggestions enumerated in the previous paragraph were followed. In the case of *Opalina,* as in that of nearly all protozoa, the first consideration is to obtain infected material. Only about ten per cent of the tadpoles examined were infected with *Opalina*. As exposure to air is harmful to most intestinal protozoa, the plan of spreading the intestinal contents on a slide for microscopic examination was abandoned early in the work. It was found best, in order to save much labor as well as to preserve the organisms in a fresh state, to place the unopened intestine on a sterile slide and then to examine it under the low-power microscope. In this way, uninfected animals were quickly discarded. In the case of *Opalina,* it has been determined by previous workers that the organism will live for days in concentrations of salt solution ranging from one-half to one per cent. In our work, it was thought best to determine just what salt concentration was most favorable to *Opalina*. In order to do this accurately, the method of Giffin and Sanford for testing the fragility of red blood corpuscles were used, with this difference: in the fragility test, concentrations of salt from 0.5 per cent down to 0.2 per cent are used; in this work with *Opalina,* concentrations from one per cent down to 0.1 per cent were used. In this way, the most favorable salt concentrations were determined within fairly definite limits. Having determined fairly accurately the correct salt concentration, the next step was to use this salt concentration with varying hydrogen-ion concentrations. As the hydrogen-ion concentration of the intestinal contents is rather definitely known, it was not necessary to cover a wide range of variations. A pH of between 6 and 8 will cover the requirements of the majority of intestinal protozoa.

In the case of *Opalina*, varying concentrations of human serum were used, from 0.1 per cent up to ten per cent. In addition, following the work of Rees, a sprinkling of rice starch was added to one set of tubes. As some organisms grow best in the presence of free oxygen, as in the upper part of the tube, and others grow best with the oxygen partially excluded, the height of the column of medium was varied from a few millimeters up to twenty millimeters. In this work, four sets of tubes were inoculated. The first set was plugged with cotton stoppers. The second set was plugged with rubber stoppers. The third set was gently heated at the open end before inserting a rubber stopper, and the fourth set was heated to a much higher temperature before inserting rubber stoppers. Three different temperatures were used: (a) the low temperature incubator or the ice-box (this temperature is about 10° C.), (b) room temperature, or a low temperature incubator (a temperature of about 25° C.), and (c) the ordinary incubator (37° C.). In the case of cold-blooded animals, room temperature, or, more preferably, ice-box temperature, is best suited to their growth. Cultures made at the warmer temperatures have to be transplanted at more frequent intervals. In the case of cold-blooded animals, the transplants may be made weekly, or even bi-weekly.

METHODS IN GENERAL USE IN THE CULTIVATION OF AN ORGANISM

In the selection of a medium for the cultivation of intestinal protozoa, several factors should be considered:

(1) The simplicity of its formula.
(2) The facility of its preparation.
(3) The availability of its various constituents.
(4) Its physical character, as determined by the ease with which portions may be removed for repeated examination.

At present, there are in general use three main types of culture media for intestinal protozoa: (1) Hogue's ovomucoid medium, (2) serum-salt solution, and (3) Boeck's Locke-egg-serum medium.

These three media are used with a number of modifications. All three, with their various modifications, give good growth of all intestinal protozoa so far cultivated, with the exception of the parasitic amœbæ. In the case of parasitic amœbæ, Boeck's medium is the medium of choice for the growth. In the hands of the writer, the serum-salt solution medium, especially with the modification of Hegner, has proven the simplest in preparation and has given the most profuse growth of any of the others tried. In addition, the precipitates formed by bacterial growth have apparently been less in quantity, and less harmful to the growth of protozoa.

CULTIVATION OF SPECIFIC ORGANISMS

The cultivation of the intestinal amœbæ is taken up in another chapter of this book. The remaining protozoa infesting the human intestine may be classified for our purposes, as, (a) those passing their entire life cycle in the intestine, and (b) those requiring an intermediate host for their complete development. Obviously, cultural methods can be applied only to the former class.

The known human intestinal PROTOZOA belonging to (a) are: (1) *Trichomonas hominis,* (2) *Embadomonas intestinalis,* (3) *Giardia lamblia,* (4) *Enteromonas hominis,* (5) *Chilomastix mesnili,* and (6) *Balantidium coli.*

Of these organisms, *Giardia* and *Enteromonas* have not been cultivated.

Trichomonas hominis was first cultivated by Lynch (1915) on a medium composed of acidified bouillon. His cultures multiplied at first, but lived only a few days. Boyd (1918) attempted the cultivation of this organism in sterile salt solution to which was added non-sterile fecal material. This is apparently an example of preserving the life of the organism, rather than cultivating it. The writer has preserved living *Trichomonas* for months in various media, keeping the cultures in an ice-box. In the hands of the writer, the most favorable medium for the cultivation of *Trichomonas* is serum-salt solution, as used by Barret and Smith (1924). Cysts of *Trichomonas* are unknown.

Embadomonas intestinalis. "Hogue (1921) was the first to cultivate this species using the ovomucoid medium described above and also a Locke-egg medium and an ox bile salt medium. Cysts appeared in tubes that contained no other protozoon; this is apparently the first authentic record of the formation of cysts by any intestinal flagellate in cultures."—Hegner and Taliaferro (1924).

Chilomastix mesnili was first cultivated by Boeck (1921) using a medium composed of human serum and Locke's solution with the addition of a small amount of dextrose.

Balantidium coli was first cultivated by Barret and Yarbrough (1921) using a medium composed of one part of human serum plus sixteen parts of 0.5% sodium chloride solution. The balantidia multiplied over a period of thirty-two days, eleven transplants being made. Recently Rees (1927) has successfully cultivated balantidia from the pig and guinea-pig using a modification of the medium of Barret and Yarbrough. Rees substituted Ringer's solution without dextrose for the sodium chloride solution and added a minute amount of rice starch.

PROBLEMS FOR FUTURE WORK

Protozoölogy, in all its numerous phases, presents a vast field for research. Of the many problems suggesting themselves in the particular field of this chapter, a few are here mentioned:

(1) *Obtaining pure cultures of the various protozoa.* Cleveland's work (1928) along this line is notable. Several problems present themselves in this particular field. First, is the obtaining of pure line cultures, or cultures from single organisms. Second, the isolation of pure line cultures and their cultivation with a single known species of bacteria. Third, the isolation of pure line cultures and their cultivation with a single known species of dead bacteria. Fourth, the isolation of a pure line culture free from all bacteria.

(2) *Infection experiments with pure cultures.* This problem would be dependent upon, to a certain extent, the problems previously mentioned. After obtaining any of the so-called pure cultures above mentioned, the next step would be the infection of protozoa-free animals with these cultures, with a view to determining (a) methods of infection, length of incubation time, length of infection, and the effect, if any, produced on the infected animals; (b) the results of various methods of treatment of the infection.

(3) *A study of the life history of the various protozoa, especially Trichomonas.* This subject is self-explanatory. Little is known of the life history of *Trichomonas*. Cysts are not known. Practically nothing is known of the mode of infection with this organism in nature. Much helpful work has been done by Hegner and others along this line.

(4) *Attempts to cause encystation of Trichomonas, using the method outlined by Cropper and Drew for free-living amœbæ.*

The work of Cropper and Drew (1914), using various "auxetics" and "kinetics" to produce encystation and excystation in free-living amœbæ, seems to offer many helpful suggestions in studying the cysts of the various protozoa and in the production of cysts in those organisms in which cysts are unknown, either in culture or in their natural habitat. This same work calls to mind many problems in the rate of multiplication, and length of life or organisms in culture. In the cultivation of an amœba from the turtle, Barret and Smith (1924), cultures which had almost completely died out after two years on artificial media were "rejuvenated" by the addition of 0.2% of choline and carried for several months longer.

(5) *The effects of various chemicals and dyes on protozoa* in vitro *and* in vivo. On the parasitic amœbæ, emetine and various other drugs have been found to exert a more or less specific effect, when administered in cases of amœbic infection. No specific drug has been found for infections with the flagellated or ciliated PROTOZOA. The treatment of infections with this class of organisms offers one of the biggest fields for research at the present time. Numerous aniline dyes, as well as various drugs, should be studied for their effects upon protozoa, both in the test tube and in the living organism.

(6) *A study of the specificity of protozoa for their original host, and for their original habitat in a given host.* Hegner (1928) has done some valuable work on this problem. When we consider the universal prevalence of *Trichomonas* and other intestinal protozoa and the great variety of animals infected by these organisms, a vast amount of work could be done to determine whether or not these organisms are specific for the animals and for the organs in which they are found. The mutations in bacterial life taking place in changed environment and in different hosts are well-known. Might we not find like changes taking place in protozoa, under changed conditions of life? This would seem a broad field for future investigation.

CHAPTER IX

TRANSMISSION OF INTESTINAL PROTOZOA

By

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INTRODUCTION

One of the most interesting phases in the life-cycle of intestinal protozoa is that during which they are transmitted from one host to another. It is at this time that measures for prevention and control can most successfully be applied, measures which, from the standpoint of personal hygiene, protect the individual from infection, and, from the standpoint of public health, protect the general population, either rural or urban, from infection. The fifteen species of intestinal protozoa that are usually recognized by protozoölogists at the present time may be classified as follows with respect to their habitat within the body and the method by which they are transmitted:

1. Species transmitted by the contamination of food or drink by cysts:
   - (a) INTESTINAL AMŒBÆ:
     - (1) *Endamœba histolytica*, the organism of amœbic dysentery and amœbic liver abscess.
     - (2) *Endamœba coli*, a harmless commensal living in the large intestine.
     - (3) *Endolimax nana*, similar to (2).
     - (4) *Iodamœba williamsi*, similar to (2).
     - (5) *Dientamœba fragilis*, similar to (2).
   - (b) INTESTINAL FLAGELLATES:
     - (6) *Chilomastix mesnili*, a possible causative organism of flagellate diarrhea.

(7) *Embadomonas intestinalis,* probably a harmless commensal.
(8) *Tricercomonas intestinalis,* also probably a harmless commensal.
(9) *Giardia lamblia,* accused of causing flagellate diarrhea.

(c) Coccidia:

(10) *Isospora hominis,* the agent of a type of diarrheic infection known as coccidiosis.

(d) Infusoria:

(11) *Balantidium coli,* the ciliate responsible for balantidial dysentery.

2. Species transmitted by the contamination of food or drink by trophozoites:

(12) *Trichomonas hominis,* another flagellate accused of causing diarrhea.

3. Species transmitted by contact in the trophozoite stage:

(13) *Endamœba gingivalis,* an amœba that lives in the mouth and that has been accused, probably unjustly, of causing pyorrhea.
(14) *Trichomonas buccalis,* apparently a harmless flagellate living in the mouth.
(15) *Trichomonas vaginalis,* a flagellate living in the vagina that may be pathogenic.

TRANSMISSION BY CONTACT

Endamœba gingivalis. The two species of protozoa that occur in the human mouth are probably transmitted by contact; that such a method of transmission is effective is indicated by the fact that a large percentage of the general population are infected. *Endamœba gingivalis* lives in the tartar of the teeth and in the materia alba around them. The exact relation between this species and the host is not known. It was once supposed to be the causative agent of pyorrhea, but is now usually considered harmless. Kofoid (1928) has recently considered this subject in some detail. The transmission of *E. gingivalis* appears to take place in the trophozoite stage only. Cysts have been described, but there is no evidence that they really exist, although Wenyon (1926) states that they "probably occur." If they do they must play a minor rôle in the life-cycle of the organism. Transmission from host to host no doubt takes place in the trophozoite stage and plenty of opportunity is afforded for direct passage during kissing. It is thus easy to account for the high incidence of infection in the general population which probably averages at least fifty per cent. This species of amœba, although in the active stage when disseminated, is probably passively carried from mouth to mouth. The absence of a cyst stage in the only amœba of man that is transmitted by direct contact is worthy of note.

Endamœbæ have been reported from the mouths of certain lower animals, including the cat, dog and horse. Attempts have been made to transmit amœbæ from the mouth of man to certain lower animals. Hecker (1916) was unable to infect guinea-pigs and Drbohlav (1925) did not succeed in obtaining infection in a young dog in the gingivæ of which *Endamœba gingivalis* grown in culture was inoculated. Hinshaw (1928), however, was successful. It has been suggested that the amœba of the mouth of man is the same species as *E. histolytica* of the intestine. The studies of Kofoid and Swezy (1924), however, indicate that this is highly improbable. If they should prove to be the same species transmission would probably be due to the ingestion of food or drink contaminated with material containing *E. histolytica.*

Trichomonas buccalis. This mouth-inhabiting flagellate is widespread, occurring in perhaps a third or more of the general population. As in the case of *E. gingivalis* and *E. histolytica,* it has been suggested that *T. buccalis* of the human mouth and *T. hominis* of the human intestine belong to the same species, the latter being specimens from the mouth swallowed by the host. Most of our evidence, however, indicates that these two flagellates belong to different species. Both Hogue (1926) and Hinshaw (1926) found that *T. buccalis* is more frequently present in the mouths of persons suffering from pyorrhea, acute gingivitis or abscessed teeth than in persons with normal mouths. Hogue also found that strains of *T. buccalis* grown in culture tubes differed with respect to their resistance to temperature and to the pH of the medium. It thus seems probable that the effectiveness of the transmission, which no doubt usually results from the passive transfer of the organisms from one host to another during kissing, depends somewhat on the resistance of the strain and the condition of the buccal cavity, whether diseased or normal.

Trichomonads have been described from the mouths of lower animals by Hegner and Ratcliffe (1927*a*, 1927*b*). A species, named by these investigators *Trichomonas canistomæ,* was reported from twenty-two of twenty-three dogs examined. Since this report was prepared the twenty-third dog and twenty-six other dogs examined have all been found to be positive; thus 100 per cent infection existed in these forty-nine dogs from Baltimore. Trichomonads were obtained also from the mouths of two cats; these have been named *Trichomonas felistomæ* (Hegner and Ratcliffe, 1927*a*). Although these two types have been given specific names there is a possibility that they may belong to one species and that this species may be the same as *T. buccalis* in man. Culture and cross-infection experiments are necessary to determine this point.

Trichomonas vaginalis. This flagellate has been reported most frequently from vaginal mucus but has also been recorded from the urinary tract of man. It appears to be present in from ten to fifty per cent of adult women (Brumpt, 1913; Hegner, 1925) and hence must find transmission comparatively easy. As no cyst stage occurs in its life-cycle it must be transmitted in the trophozoite condition. How *T. vaginalis* reaches the vagina is uncertain. Specimens from the vagina may easily gain access to the urinogenital tract of men during coitus. The vagina may become infected during coitus but this has still to be proved. Contamination with specimens from the intestine or during homosexual practices are also possibilities (Dickinson and Pierson, 1926). It seems probable that the incidence of infection among men is higher than reports now available indicate. The exact distribution of these flagellates in the various parts of the urinogenital tract is not known.

The only lower animals in which vaginal trichomonads have been reported are monkeys belonging to the species *Macacus rhesus*. Hegner and Ratcliffe (1927) have given the name *Trichomonas macacovaginæ* to this form, although further studies are necessary to prove conclusively that it differs from the trichomonad that lives in the intestine of the monkey. The writer (Hegner, 1926) has been unable to find trichomonads in the vagina of a number of domesticated animals that have been examined, including 35 sows, 100 cows, 103 calves and 108 sheep. Attempts to transfer human vaginal trichomonads to dogs, rabbits and guinea-pigs have been unsuccessful (Blockmann, 1884; Dock, 1894).

TRANSMISSION OF *Trichomonas hominis* IN THE TROPHOZOITE STAGE

No cysts are known in the life-cycle of this intestinal flagellate, hence it must be transmitted in the trophozoite stage. The only conceivable method of reaching the intestine appears to be by the fecal contamination of food or drink that is ingested by the host. The first question that arises is whether trophozoites are able to withstand the action of the digestive juices during their passage through the stomach and small intestines. It has been proved that *Trichomonas muris* is able to do so in the rat and there is every reason to believe that *Trichomonas hominis* can in man (Hegner, 1924). Material containing *T. muris* was taken from the cecum of the rat, diluted with normal saline solution and injected into the stomach of other rats by means of a hard rubber catheter attached to a syringe. These rats were killed at intervals and it was found that trophozoites of this species are capable of remaining actively motile for at least one hour after being injected into the stomach of the rat; that they may pass from the stomach into the duodenum apparently unharmed within half an hour; that they may reach the cecum through the stomach and 780 millimeters of small intestine within half an hour and still be actively motile; and that a rat free from trichomonads may acquire a cecal infection within four days after trophozoites are injected into the stomach. Later experiments (Hegner, 1926) prove that the trophozoites of *Trichomonas caviæ* and *T. flagelliphora* of the guinea-pig are able to pass through the stomach and small intestine of a guinea-pig and reach the cecum apparently unharmed within an hour after being injected into the stomach. Other investigators have recently confirmed these results. Thus Brumpt (1925) has reported the infection of cats by the ingestion of trophozoites of *Trichomonas felis* and Wenrich and Yanoff (1927) have shown that four species of rat trichomonads and one species from man may likewise be infective to rats in the trophozoite stage.

The next problem is whether *Trichomonas hominis* is able to live outside of the human body in fecal material long enough for food and drink to become contaminated. A number of experiments bearing on this problem were carried out by the writer (Hegner,

1928) and demonstrate that when fecal material containing trophozoites of *T. hominis* is kept at room temperature (about 21°C. = 70°F.) no conspicuous diminution in the number of flagellates occurs for the first few hours, but after forty-eight or seventy-two hours very few remain alive; these persist for as long as eight days. Sufficient time is thus allowed for the transfer of viable specimens to the food or drink of man. Feces, containing large numbers of *T. hominis,* were deposited on garden soil in the shade during a wet, cloudy period when the temperature ranged from 12°C. (54°F.) to 19°C. (66°F.). Only a slight decrease in numbers of flagellates was noted during the first twenty-four hours, but very few were present at the end of seventy-two hours. Cultures of serum-saline-citrate medium inoculated with samples from the fecal mass were positive on the fourth, fifth and seventh days. A similar stool, when deposited on sandy soil, sank below the surface almost immediately. Positive cultures were obtained with contaminated sand at the end of twelve hours, but not later. The character of the soil is thus of considerable importance in the viability of *T. hominis* in localities where soil pollution is prevalent.

When a sample of fecal material containing *T. hominis* is stirred up in a drop of tap water the flagellates become spherical almost immediately, lose their power of locomotion and change from a bluish green to a transparent appearance. No changes are visible when similar material is stirred up in saline-citrate solution. These effects are attributed principally to changes in the osmotic pressure of the medium. The osmotic pressures of the various materials used as determined by the freezing-point depression method, were as follows: undiluted feces, 6.145, serum-saline-citrate medium, 7.710, saline-citrate solution, 7.469, ten grams of feces in ninety cubic centimeters of tap water, 0.603, five grams in ninety-five cubic centimeters, 0.482, and one gram in ninety-nine cubic centimeters, 0.121. It, therefore, seems evident that there is plenty of opportunity for trichomonads to reach new hosts in a viable condition but that there is very little danger of infection due to contaminated drinking water since the flagellates are killed very quickly in this medium.

Experiments by the writer (Hegner, 1928) and others indicate that flies may serve as important transmitting agents of *T. hominis* but that cockroaches probably do not, since most of the trichomonads are destroyed in the crop within from two to five and one-half hours; very few flagellates reach the stomach alive, and passage through the stomach and intestine is so slow that it is highly improbable that any specimens, in a viable condition, are ever deposited in the feces.

Flies of three species were fed human feces containing *T. hominis*. Living trichomonads were recovered from the intestine of *Cynomyia cadaverina* from twenty-five minutes to five and one-half hours after ingestion, from *Musca domestica*, one and two hours after ingestion, and from *Lucilia sericata* two and three-quarters hours after ingestion. When inoculated into serum-saline-citrate medium positive cultures were obtained with fecal material ingested by *Cynomyia cadaverina* from twenty-five minutes to three hours previously and by *Musca domestica* one and two hours previously.

Flies of the same three species were fed human feces infected with *T. hominis* and watched until they vomited or defecated. The vomita or feces from the flies were then examined and living trichomonads were found in droppings at various intervals between twenty minutes and six hours after ingestion. Positive cultures were obtained from droppings at intervals of from twenty minutes to four hours after ingestion. These results strongly favor the hypothesis that flies play an important rôle in the dissemination of *T. hominis*.

The host-parasite specificity of the intestinal trichomonads of man and lower animals has an important bearing on the transmission of these organisms. Trichomonads are known to occur in practically all of our domesticated mammals and birds as well as in various species of cold-blooded animals with which human beings are more or less associated. Separate specific names have been applied to the trichomonads in these various species of hosts but it is impossible to separate many of them on the basis of morphological differences and cross-infection experiments must be carried out before their specific identity is definitely established. An amazing situation was discovered by the writer (Hegner, 1929*b*) with respect to the infectivity of the chick. It was found that trichomonads from the mouth and intestine of man, from the intestine of monkey and pig and the cecum of the rat, guinea-pig and prairie dog, when injected into parasite-free chicks either by mouth or by rectum, grew and multiplied readily in the ceca of the chick and set up infections there that remained for periods of from several days to over six months. These results indicate that the chick may be an important transmitting agent in regions where sanitary conditions are not satisfactory and suggest that other domesticated animals may also transmit various species of *Trichomonas*. There is still much work of this type to be done.

TRANSMISSION OF INTESTINAL PROTOZOA IN THE CYST STAGE

This is the most common method of transmission and is the way in which eleven of the fifteen human intestinal species succeed in invading new hosts. The various percents, in round numbers, of the general population that are estimated to be infected by these organisms, which give some idea of the effectiveness of this method of transmission, are as follows (the number of infections reported for those species for which percents are not given is too small to furnish reliable figures):

	Percent
Endamœba histolytica	10
Endamœba coli	50
Endolimax nana	25
Iodamœba williamsi	10
Dientamœba fragilis	10
Chilomastix mesnili	10
Embadomonas intestinalis	—
Tricercomonas intestinalis	—
Giardia lamblia	15
Isospora hominis	—
Balantidium coli	—

There is some evidence that racial differences exist with respect to susceptibility to infection with the various species listed; and there is no doubt that age has a distinct influence, since children are more often infected than adults. The success of transmission depends to a considerable degree on the number of cysts discharged by the hosts and the ability of these cysts to live outside of the body until they are ingested by new hosts.

Endamœba histolytica. We know more about the transmission of this species than of any other. The reader is referred to pp. 65-83 of the writer's book on *Host-Parasite Relations between Man and His Intestinal Protozoa* (Hegner, 1927) for a detailed discussion of this subject. One question of interest is that of the possibility of infections as a result of the ingestion of trophozoites. Experiments with animals indicate that trophozoites may be infective but ordinarily are not infective in nature. Another problem involves the infectivity of immature cysts. When cysts of *E. histolytica* escape from the body they possess one, two, three or four nuclei. Those with less than four nuclei are immature. Do these continue their development and become infective? Yorke and Adams (1926) have shown that immature cysts continue their development in artificial culture media. This suggests that all cysts may be infective regardless of the number of nuclei they contain.

There is no question but that a sufficient number of cysts are formed in *E. histolytica* carriers to account for the incidence of infection in the general population; for example, it has been estimated that a single person may pass several hundred million cysts in a single day. These cysts, however, must be able to withstand various environmental factors outside of the host until they are ingested by new hosts. One of these factors concerning which there can be little doubt is that of desiccation. Cysts when dried are killed and hence distribution of viable cysts in a dried condition in dust appears to be impossible.

Various methods of determining whether cysts are alive or dead have been devised. One of these is the eosin test. Eosin in a concentration of 1 : 1000 is added to a smear and is supposed to penetrate and stain dead cysts but not those that are alive. This test is not entirely satisfactory since dead cysts are not always stained. Neutral red and Congo red have also been employed but are both open to the same objections as is eosin. The best method of determining whether cysts are alive or dead is to test them in susceptible animals or in artificial culture. The culture method perfected by Boeck and Drbohlav (1925) and modified by Dobell (1928) and others enables one to inoculate media with material and to determine with certainty whether living specimens of *E. histolytica* are present. If positive cultures result there can be no question but that living specimens were present. However, living specimens may have been present even if they do not appear in the cultures since not all trophozoites or cysts continue to live and reproduce in culture media.

The viability of cysts can also be determined by injecting them into laboratory animals as described in Chapter XXI. Kittens are the most susceptible of all available animals for this purpose. The experiments of Sellards and Theiler (1924) with kittens indicate that cysts of *E. histolytica* are still infective after six days when left in fecal material at 2° C., but lose their infectiveness after two weeks. Walker and Sellards (1913) were able to infect man with cysts of *E. histolytica* after they had been outside the body in fecal material for two days at tropical temperature and with cysts of *E. coli* after ten days under similar conditions. It seems certain from this that cysts of these two species are able to live outside of the body long enough to become disseminated and to find their way into the food or drink of human beings.

Cysts of *E. histolytica* are not only able to live at room temperature (21° C.) but at lower temperatures. Whether they could survive winter conditions in temperate regions has not been determined. Boeck (1921) found that all cysts of *E. histolytica* were killed at 68° C. when held at that temperature for five minutes. Yorke and Adams (1926) put 50° C. for five minutes as the lethal temperature for these cysts but found that they would withstand a temperature of 45° C. for thirty minutes. It seems probable that the organisms in the cyst stage are able to withstand ordinary temperature conditions in tropical and sub-tropical regions and all but the most severe winter conditions in the colder parts of the world so long as they are kept moist. Further experiments with temperatures under various conditions are desirable both from scientific and practical standpoints.

Various investigators have attempted to determine the effects of chemicals on protozoan cysts with a view to the disinfection of fecal material. Among these are Kuenen and Swellengrebel (1913), Wenyon and O'Connor (1917) and Bercovitz (1924). A simple method seems to be the addition of cresol in the strength of 1:20 to the fecal material. It is obvious that a thorough mixture must be made in order to reach all of the cysts. The practical value of this method is still to be determined. We are in need also of a practical method for sterilizing drinking water. It is probable that the pasteurization of milk raises the temperature to a high enough point (60° C.) for a sufficient time to destroy any protozoan cysts present and of course the boiling of water quickly kills all cysts. Mills, Bartlett and Kessel (1925) have concluded from their experiments that "Dipping fruits and vegetables for ten seconds in boiling water, or water which remains above 80° C. during immersion, is the only method thus far discovered which will uniformly kill all pathogenic bacteria, protozoan cysts and helminth eggs which might be found contaminating such food products, and render them safe for human consumption in an uncooked condition."

Both infective cysts and infective trophozoites of intestinal protozoa are passive during transmission and man is responsible for his own infection. Many opinions have been expressed regarding the method of entrance of cysts into the mouth of man. It seems probable that cysts reach the mouth in contaminated food and drink but they might also find their way there on soiled hands. Among the most important factors that bring about the dissemination of cysts, and especially their presence in food and drink, are probably the handling of food in homes, restaurants, hotels and markets by infected persons who are passing cysts; the use of night soil as fertilizer in vegetable gardens; the common use of toilet, washbowl, and towel; and the presence of insects, such as flies, ants and cockroaches, and of domestic animals, such as rats, mice, dogs and cats.

No one has yet determined how effective these various factors are in the dissemination of protozoan cysts. Perhaps the work with flies has been carried out more carefully than that with any other factor. The data obtained by Wenyon and O'Connor (1917), Buxton (1920), Root (1921) and others seem to prove conclusively that flies both in the laboratory and in nature ingest fecal material containing protozoan cysts; that the cysts are not quickly killed in the flies' intestine; that living cysts may be deposited in the feces or vomita of the fly from five minutes to forty-nine hours after feeding; and that these cysts may be deposited in the food or drink of man where they may remain viable until ingested by a human host. The conclusion seems inevitable that flies play an important rôle in the dissemination of *histolytica* cysts and that food and drink should be protected from their visits, especially in localities where amœbic dysentery occurs and sanitary conditions are such as to allow flies access to infected fecal matter. It may be pointed out here that the fly does not become infected but is a "passive" carrier, in contrast to the rat described below which is an "active" carrier.

Among the animals that have been found to be infected with *E. histolytica* in nature are cats, dogs and rats. Apparently cysts are not developed in either the cat or the dog, hence there is little danger of transmission as a result of infections in these animals. The experiments of Kessel (1923) and Chiang (1925), however, indicate that rats may become infected and pass cysts of *E. histolytica*. Under favorable conditions rats might become infected in nature but how important a rôle they play in the dissemination of *E. histolytica* cysts is unknown.

Comparatively little experimental work has been carried on with cysts of the other intestinal protozoa. It seems probable, however, that they are subjected to the same factors as are those of *E. histolytica* and are transmitted in much the same way. Many of the investigators who carried out experiments with the cysts of *E. histolytica* used also the cysts of other intestinal protozoa and their findings may be found in their publications. The cysts of the human coccidium, *Isospora hominis,* appear to be more resistant to conditions outside of the body than are the cysts of the intestinal amœbæ and flagellates. Haughwout (1921), for example, exposed oöcysts in the sporoblast stage to the sun for three hours every day for a week and found that at the end of this period some of them developed sporozoites when water was added; and Wenyon (1926) reports the completion of development within the oöcysts of *Eimeria stiedæ* from the rabbit after being subjected to a fixing solution of sublimate, stained with hematoxylin, dehydrated, cleared, and mounted in balsam.

Of particular interest is the situation regarding *Balantidium coli* (see Chapter XXIV). Cysts of this species seem to be rare in man, not so rare in monkeys, but more common in pigs. A careful study of cyst production in these three types of hosts, as well as in guinea-pigs, and the resistance of these cysts to factors in the environment outside of the body would well repay investigation.

On the whole it seems to the writer that our knowledge of the transmission of intestinal protozoa, which is so important from a public health standpoint, is based on fragmentary and in many cases uncontrolled experiments. Furthermore, most of these experiments have been conducted in the laboratory and in many cases do not represent satisfactorily conditions as they are in nature. We have here an important field for investigation in which careful work would furnish results of considerable importance.

CHAPTER X

METHODS AND PROBLEMS OF CROSS-INFECTION EXPERIMENTS WITH INTESTINAL PROTOZOA OF MAN AND OF CERTAIN LABORATORY AND DOMESTIC ANIMALS

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INTRODUCTION

With the discovery that most of the common laboratory and domestic animals harbor intestinal protozoa similar to those found in man there has been much speculation as to whether animals could serve as reservoir hosts in the transmission of the intestinal protozoa to man and also as to whether the species of intestinal protozoa of man are identical with or different from those found in the lower animals. Similar questions obviously arise with reference to the interrelationships of the intestinal protozoa of the various lower mammals.

Many of the opinions stated in the past have been formed without resorting to adequate experimental measures and much discussion that has taken place reminds one of the debates reputed to have occurred during the Middle Ages concerning the number of teeth possessed by the horse, all without the participants having resorted to the simple measure of counting the horse's teeth. It is obvious, of course, that the difficulties encountered in the problems under present consideration are not to be overcome so easily but will require the collection of much data by tedious experiments.

As a consequence of the speculation on this subject in the past two contrasting views have become evident. The first assumes that the intestinal protozoa of man and many of the lower animals with which he is associated are identical, that the host-parasite relationships are not rigid and that the lower mammals may serve as reservoir hosts for human intestinal protozoan infections. The second hypothesis concludes that the intestinal protozoa of man and the lower mammals represent different species, that the host-parasite relations are rigid and that the lower mammals may not serve as reservoir hosts for human intestinal protozoan infections.

Obviously the earlier view was formulated as the result merely of observing the presence of intestinal protozoa of the lower animals similar in appearance to those seen in man. Because they presented related morphological characteristics they were held to be the same species. As a rule little or no experimental work was carried out before conclusions were drawn. Later, certain investigators resorted to the experimental method to support their contentions but the experiments were usually carelessly conducted, without adequate controls and with insufficient care in the selection of parasite-free animals before the transmission experiments were carried on.

With evident differences in morphology, which became apparent as the result of investigations by the cytologist, certain species came to be clearly differentiated on the basis of morphology. This gave the necessary stimulus to unlimited species differentiation which, coupled with a few negative results obtained from inadequate transmission experiments, led to the formation of the second or negative view which assumes, purely on the basis of host environment, that parasites within different species of mammals are different.

To hold rigidly to this second and restricted contention is just as unfortunate as to accept the first extreme view, for it seems probable that each species of host and of parasite must be considered on the basis of its own individual relationships. Hence a third or intermediate view, which is the one held by the writer, is apparent. This may be stated as maintaining that certain parasites are non-rigid and can be transferred with facility from host to host while others may be found to be restricted to individual hosts.

The factors involved in determining these relationships are as yet quite obscure but it seems probable they are mostly biochemical in scope. Variation in hydrogen-ion concentration in the intestine; variation in type of diet, *i.e.,* whether carnivorous, vegetarian or omnivorous; or whether of high or low protein content; and the type of vitamines present, all appear to be of importance in such determinations. The type and strength of digestive secretions present are undoubtedly also factors and it seems safe to assume that symbiotic relations with bacteria and intestinal yeasts may also be of importance in determining relationships. The habitat of the parasite within the host also may influence rigidity, *e.g.,* it seems probable that a parasite which has adopted an intra-cellular mode of existence, *e.g.,* a coccidium, may possess a more restricted host-parasite relationship than one which lives almost entirely within the lumen of the intestine and penetrates only between cells, for we know pH within the cell has very narrow limits while the pH of the fecal contents of the intestine may vary from 8.0 to 5.0.

The problems involved in carrying on investigational work on the subject of cross-infection and host parasite relationships are legion and the conclusions drawn as a consequence of the observations are related to a variety of biological fields.

These investigations are of interest chiefly from (a) the theoretical point of view, involving questions of taxonomy and evolutionary significance; (b) the point of view of sanitation and public health involving questions of reservoir hosts which in turn are dependent upon host-parasite relations; (c) the point of view of experimental biology and medicine with special reference to experimental pathology, histology and therapeusis.

In order to study the extent of a disease it is important that a suitable experimental animal be procured. Such procedure involves acquainting oneself with the natural infections of the animals to be employed in the experimental work, *e.g.,* if one is to attempt transfer experiments with rats and monkeys one must first become thoroughly acquainted with the intestinal fauna of the rat and monkey and ascertain wherein the natural protozoa of the rat may differ from those of the monkey.

The question of a satisfactory basis for species differentiation is one on which thorough agreement has not been reached by protozoölogists. It seems to the writer, however, that such differentiation should be based on the following determinations:

(1) Morphological or cytological differences which are constant. The chief pitfall in species differentiation among protozoa on the basis of morphology is in mistaking physiological variations and mitotic stages for constant morphological differences.

(2) Reactions and growth in culture. Cultural modifications for species determination among the protozoa have not been

worked out in great detail as yet but the L.E.S. medium of Boeck and Drbohlav (1925) with its modifications is invaluable.

(3) Evidences of host-parasite rigidity or transferability based on animal transfer experiments.

(4) Comparison of the pathological results produced by the parasite in the natural host and in the hosts to which the parasite has been experimentally transferred. In this respect a common animal in which the pathological conditions can be compared or standardized is important, *e.g.,* the kitten is so susceptible to acute amœbiasis that this animal has come to be recognized as a standard animal for the study of acute amœbic infection.

Most new species names which have appeared in the literature have commonly been given without the application of the above standards of species differentiation. As a matter of fact, it has become quite the habit of some protozoölogists of late to name new species of protozoa, even when their morphology is identical, purely on the basis of their being found in a host from which they have not previously been reported. This is particularly noticeable with reference to the *Endamœba* which forms eight nucleate cysts and resembles *Endamœba coli* in man. An amœba of this type has been found in man, several species of lower primates, several species of rats and mice, the rabbit, the guinea-pig, the cow, the domestic pig, the ground squirrel, the goat and sheep. In most cases new species names have been given to these amœbæ and these without adequate study, the conclusions being drawn purely on the basis of the fact that the amœbæ were found in a host from which they had not previously been recorded. This has been done in spite of the fact that nearly every writer acknowledges that he can see no morphological difference between his new species and *Endamœba coli* of man. It is apparent that we must resort to methods of differentiation more critical in character than a new host environment alone if these names are to be justified.

The same problem has also been encountered in respect to the amœba resembling *E. histolytica* of man, forms exhibiting similar morphological characteristics having been found in several species of monkeys, the rat, in the domestic pig and in naturally infected kittens. The morphological appearance of these amœbæ is identical; they grow in L.E.S. culture medium; they produce similar pathological symptoms in kittens experimentally infected; and sufficient animal transfer experiments have been accomplished to indicate that this amœba is interchangeable. The only logical conclusion in the light of these data is that these amœbæ of man, of the monkey, of the rat and of the domestic pig all belong to the same species and that they have merely gained access to different hosts. It may of course be found that cultural or physiological differences, similar to those employed in differentiating bacteria, will give more delicate means of species differentiation among the protozoa than we now know. In such event these amœbæ of animals which are morphologically similar to the amœba of man may exhibit serological differences which will warrant their being classified as different species.

In all probability all amœbæ that exhibit similar morphology have a common origin and the problem left with us is to determine whether the environments of the digestive tracts of different animals present a sufficiently wide variation in biochemical range to have altered individual parasites sufficiently to restrict them to a specific host.

METHODS IN GENERAL

Experimental cross-infection work in relation to the problems mentioned above must all be accomplished with the lower animals since man cannot be used safely for experimental infections on account of the dangers associated with his acquiring protozoan infections and bacterial infections of uncertain pathogenicity that might be transmitted to him during the experiment.

Animal transfer experiments, however, are imperative as aids in determination of species identity and to problems in host-parasite relationships. These must be performed with the greatest caution and the necessity of procuring absolutely parasite-free animals for experimental work cannot be too greatly emphasized.

A. Selection of Negative Animals

Animals negative for parasitic infection may be selected by the following methods:

I. Examination of feces for the detection of intestinal protozoa. Feces for examination may be collected in a variety of ways.

(a) *Collection of normally passed feces.* As a general rule this method may be held to be more suitable for animals that normally have only one or two evacuations daily than for animals that pass small pellets of feces frequently, *e.g.*, rats and mice. Experience in determining the presence of intestinal protozoa in man has taught us that cysts and trophozoites do not appear at regular intervals in the feces and has shown that repeated examination is imperative in order to demonstrate infections. This same fact holds true with the lower mammals.

(b) *Collection of feces following a purge.* This method has been found valuable in routine laboratory diagnosis for human protozoa. It has been shown to hasten the diagnostic procedure and to insure efficiency. Two types of purge may be given, one a mild laxative which should be given on several consecutive days. This does not often increase the number of stools per day but merely softens the fecal matter. It will be found most valuable when it is impossible to collect all stools passed. The other method is to examine stools following the administration of a drastic purge. The stools by this method are liquid and unless one can be assured that portions of all stools will be collected for examination there is the possibility that light positive infections will be missed because the protozoa if present only in a few small areas may be flushed out in an unexamined portion of the stool. When animals are kept in individual cages of such construction that all feces passed can be collected in trays underneath the cages this method is valuable. The writer has employed it successfully with rats, monkeys, kittens, and dogs.

(c) *Collection of material by enema.* This method is suitable for some animals but does not appear to yield satisfactory results in others. In animals with a large cecum, *e.g.,* the pig, or with the cecum almost entirely closed off by the cecal valve, *e.g.,* the rat, it is not as satisfactory a method as with those animals that do not exhibit a pocket type of cecum. It is obvious that the enema method is of value in detecting positive fecal material in the colon only, since little or no material will be obtained from the cecum by this method.

(d) *Collection directly from cecum by surgical method.* Ratcliffe (1928) has collected cecal contents directly from the cecum by operation for his bacterial and protozoal counts in diet experiments. This method also lends possibilities for use in the detection of protozoan negative animals but quite evidently requires further careful observation on this point before it can be used with safety. It is possible that the collection of material from a single isolated region is not sufficiently representative of the whole colon and cecum to warrant the assumption that an animal is actually negative when found to be negative by this method.

(e) *Treatment method followed by repeated examination over extended periods.* This method may be resorted to in extreme cases when negative animals are not obtainable. It, however, has certain drawbacks, first the difficulty in assuring a complete cure of the animal, there being the possibility that an infection thought to be cured will flare up again and second the possibility that animals once infected have established at least a partial immunity to a second infection. Negative experiments when animals of this type are employed would not afford final evidence that parasites were not transferable. Further, the importance of using young animals in cross-infection experiments should be pointed out. Older animals are, as a rule, more difficult to infect and often appear to exhibit an immunity to infection.

Material collected in the above ways should be subjected to examination by the following methods:

(a) Preliminary examination of smears in Donaldson's iodine-eosin stain.
(b) Examination of smears permanently stained in Heidenhain's iron hematoxylin.
(c) Study of cultures made in L.E.S. or R.E.S. medium of Boeck and Drbohlav, or suitable modifications of the same.
(d) Cultures for bacteria should also be made to rule out the presence of pathogenic bacteria when the question of pathogenicity is involved.

II. Breeding of laboratory stock from parasite-free parents. A negative pregnant female may be selected and the young kept under conditions which render contamination impossible. In order to insure definitely that the mother was negative she may be sacrificed when the young are old enough to wean, and her intestinal contents examined carefully by smear and cultural methods.

At this point the necessity of isolation rooms for each type of experiment should be mentioned. These should be so constructed that they are insect proof. It seems superfluous to mention the necessity of care in restricting utensils to individual cages, in feeding uncontaminated food and in washing hands when passing from one isolation room to another. It is safest to have only one type of experiment in progress at one time and thus minimize the danger of contamination from other sources.

In summarizing it may be stated that negative animals can be selected only by employing the greatest care and that a combination of the above-mentioned methods is often desirable. The importance of building up a stock of negative animals for experimental purposes is recommended.

B. Methods of Infection

After negative animals have been determined the question at once arises as to the best methods of infecting the animals. This will depend on the animal in question but should be accomplished by the most natural procedure. When one is endeavoring merely to establish infection, the maximum effort may be made. Since the viability and infectivity of the protozoa in question are important factors and since we are not sure of the maximum conditions for the same, *i.e.,* whether encysted protozoa require a certain incubation period outside the body before they will reach their maximum infectivity to other animals, it is advisable to collect ample amounts of feces containing cysts, feed some as soon as possible after the collection is made and allow the remainder to stand at room temperature and feed more of the material at intervals of one, two, or three days for a week. If no particular desire is present to establish an infection from a single case, protozoa from several cases may be mixed and fed.

The simplest and most natural method of infection is to mix the infective material with the food of the animal to be infected. When several animals are present in a single cage, however, it is not always possible to be sure that all animals will acquire a uniform amount of the material to be infected. Also, if spread upon or mixed with food some of the cysts may be killed by drying before being ingested. Consequently the writer in most cases has adopted the procedure of giving to individual animals by a Luer syringe to which a rubber catheter is attached, uniform amounts of dilute material previously passed through a wire screen to remove coarse débris. It takes two people to perform a satisfactory feeding by this method. The animal must be held firmly and the person holding it must grip the jaw firmly with thumb and index finger of one hand to hold upper and lower jaws of animal apart and keep the teeth from cutting the catheter. The catheter should be passed down the esophagus slowly until its entrance to the stomach is insured. Care must be taken that it does not enter the trachea. For this reason as large a catheter as can be passed comfortably should be used. Once the tube is in position, the syringe without plunger is inserted and filled with the required number of cubic centimeters of dilute fecal matter. The plunger is then inserted, and the infective material forced slowly into the stomach. When feeding material from culture, *e.g.*, *Trichomonas,* it may not be necessary to use the plunger as the culture fluid often passes to the stomach directly without force. By this method regurgitation is prevented and a uniform amount of material is assured each animal. When infection is attempted by rectum the French catheter and Luer syringe may also be employed.

It is generally assumed, though not definitely proved, that encysted stages, rather than the trophozoite stages of the intestinal protozoa of man, are the usual stages that function in establishing infection. Excepting *Trichomonas,* cysts, then, will ordinarily be given by mouth, and trophozoites by rectum. Plugging the rectum with a celloidin plug and bowel ligation to induce stasis has been used with success by a number of investigators in infecting kittens with amœbæ, but this method is quite unnatural and should not be employed in attempts to establish natural host-parasite relationships between animals.

SPECIFIC PROBLEMS AND METHODS

The animals thus far used in animal-infection experiments by the writer are listed below. The infections found in these animals, together with the methods employed in working with each animal and certain current problems related to each, are discussed.

I. RATS AND MICE

Endamœba muris, Grassi, 1881, was the first amœba described from rats and mice. Wenyon (1907) gave us the first careful description of this amœba. Brug (1919) later records finding it in wild rats in Java and discusses its morphology. Hegner (1926*a*) describes trophozoites of *E. dipodomysi* from the kangaroo rat. Kessel (1924*a*) noted the similarity of the amœba of the rat to *Councilmania lafleuri* (Kofoid and Swezy, 1921) in that the trophozoites form hyaline pseudopodia, the nuclei in the resting stage exhibit dispersed karyosomes and buds are produced when the amœbæ excyst. He transferred the amœbæ forming hyaline pseudopodia, producing eight nucleate-cysts and known as *E. muris,* Grassi, 1881, and *E. decumani,* Rudovsky, 1921, to the genus *Councilmania* and described *C. muris* as showing greatest dispersion of the karyosome and *C. decumani* as showing least dispersion of the karyosome in the resting stage.

In the light of recent experience, however, I am inclined to consider *C. muris* and *C. decumani* as representing division phases of the same species, and consider *C. decumani* as a synonym of *C. muris.*

Another form with granular pseudopodia was noted and was named *Endamœba ratti.* The cyst of this amœba answered to the common description of *E. coli* in that the karyosome of the resting nucleus was massed and excentric.

Hegner (1926*a*) found no cysts of the amœba he named *E. dipodomysi,* but the nuclei of the trophozoites he figures resemble amœbæ which produce eight-nucleate cysts more than nuclei of amœbæ which produce four-nucleate cysts. Since this amœba produced hyaline pseudopodia it appears to be more closely related to *C. muris* than to *E. ratti. E. dipodomysi* should not be accepted as a good species until cysts are described and until adequate animal transfer experiments have been performed, proving that the host-parasite relation between this and other amœbæ of rats is rigid.

The genus *Councilmania* is still held *sub judice* by some protozoölogists who consider that the hyaline pseudopodia described for *Councilmania* and the granular pseudopodia ascribed to *E. coli* represent mere physiological phases of the same amœba. The dispersed condition of the karyosome, ascribed to *Councilmania,* is also regarded by some merely as a mitotic phase of the excentric massed karyosome described for *E. coli.* Budding, through the cyst wall, previously described as a generic character for *Councilmania,* does not appear any longer to be restricted to *Councilmania,* for a similar phenomenon has recently been described for *E. histolytica, Iodamœba,* and the author has seen it in *Endamœba coli.* Thus, whether these buds be artifacts as Wight and Prince (1927) hold, whether they represent phases of a single escaping amœba through a pore in the cyst wall as recently described in detail for *E. histolytica* by Dobell (1928), or whether they represent escaping amœbulæ as was originally described by Kofoid and Swezy (1921), is of small consequence. The important facts are that such a phenomenon interpreted by some as budding, does exist, even though it is interpreted differently by other writers, and that it is not restricted to a single genus.

The characteristic dispersion of the karyosome in *Councilmania* is the most distinctive, constant characteristic of this amœba, and such dispersion is as a rule associated with trophozoites exhibiting hyaline pseudopodia, while cysts exhibiting massed excentric karyosomes described for *E. coli* are, as a rule, associated with trophozoites which form granular pseudopodia.

If the differences in karyosome and pseudopodial formation given for differentiating *C. lafleuri* and *E. coli* are accepted, then *C. muris* and *E. ratti* will be similarily differentiated. If, on the other hand, the differences between *E. coli* and *Councilmania lafleuri* are eventually decided to be only racial in character rather than generic, then *C. muris* and *E. ratti* become synonyms of *Endamœba muris*. Further, the possibility must be considered that the host-parasite relations of these amœbæ of the rat and of man are non-rigid. Kessel (1923*a*) established infections of *E. coli* and of *Councilmania lafleuri* in rats and therefore indicates this possibility. If such be true and the morphological differences ascribed to *E. coli*, Lösch, 1875, *E. muris*, Grassi, 1881, *E. decumani*, Rudovsky, 1921, *E. ratti*, Kessel, 1924, and *E. dipodomysi*, Hegner, 1926, be insufficient to differentiate them into species, then the last four become synonyms of *E. coli*. Further morphological and experimental observations are needed before such conclusions can be definitely made.

Endamœba histolytica

Lynch (1915) records an epidemic of amœbic dysentery in rats in South Carolina. His differentiation between the amœba natural to the rat and *E. histolytica* was not clearly made. Consequently there is some doubt as to whether he was actually dealing with *E. histolytica*. Brug (1919), however, records and figures an amœba from a wild rat in Java which appears without doubt to possess the same morphological characteristics as *E. histolytica* of man. Chiang (1925) in Harvard also records in rats natural infections of an amœba morphologically identical with *E. histolytica* of man. Kessel (1923*a*) was able experimentally to infect rats with *E. histolytica* of man and Chiang (1925) did the same thing. He, however, carried the experimental work farther, recovered the amœbæ from the rats and infected kittens with the same, producing an acute amœbiasis like the infection produced in kittens infected with *E. histolytica* of man. He also produced the same type of acute symptoms in kittens infected with *E. histolytica*-like amœbæ from naturally infected rats.

He does not consider these amœbæ from naturally infected rats to be morphologically or physiologically different from *E. histolytica* of man and proposed *E. histolytica* variety *murina* as a terminology for differentiation. This use of a varietal or racial term to indicate the host in which a parasite is found, was previously suggested by Chandler (1923) and appears to be a very satisfactory solution to a difficult problem when actual morphological differences are apparent. When morphological differences are not apparent, however, this system could not be used, since the trionym indicates well-established morphological differences and cannot be used to indicate merely a difference in environment as would be indicated by adding a host's name to a parasite morphologically indistinguishable from another.

This difficulty might be overcome by the use of the Latin *ex* plus the common name of the host or the scientific name of the host in which the parasite was found, *e.g.,*

E. histolytica ex mure—the type found in rats and mice
E. histolytica ex macaco—the type found in *Macacus* monkeys
E. histolytica ex sue—the type found in the domestic pig.

To clutter up the literature with an unlimited number of new species names just for the sake of having one's own family name appear after them does not seem to be justifiable and the only possible excuse for such a habit is the second one given by Hegner (1927), *i.e.,* "because of the value of a specific name for reference purposes." The suggestion made above appears to be preferable to naming new species of parasites indiscriminately merely on the basis of finding them in a new host as has been the practice adopted by some authorities. It retains the advantage of association of a parasite with a definite host and at the same time indicates a definite morphological similarity to a known type.

Iodamœba

Natural infections of rats with *Iodamœba* have not been reported. Kessel (1923*a*) and Smith (1928), however, record having infected rats experimentally with *Iodamœba williamsi* of man.

Endolimax

Chiang (1925) reports the presence of an amœba similar to *E. nana* of man in naturally infected rats. He names this amœba *Endolimax ratti*, basing his differentiation on the grounds that he did not succeed in infecting rats with *E. nana* of man. Kessel (1923*a*) failed to find natural infections of *E. nana* in rats although he did find it in rats fed *E. nana* of man experimentally. Since the flagellates of rats and their morphological characteristics are discussed at length by Dr. Wenrich in this monograph, they will not be discussed in detail here. It seems apparent, however, that certain of the flagellates present in rats, *e.g., T. muris*, exhibit morphological characteristics quite different from those found in man. Others, however, are similar to protozoa found in man, *e.g., T. minuta* is similar to the triflagellate *Trichomonas* of man (Faust, 1921), *Giardia simoni* (Lavier, 1924) similar to *Giardia lamblia* and *Chilomastix* similar to *Chilomastix* of man.

The problem of procuring rats negative for protozoa is one which is difficult. Brug (1919) mentions the irregularity with which cysts of protozoa appear in the normally formed feces. Examination of normal feces alone is considered tedious and unreliable.

Kessel (1923) endeavored to establish a suitable method by examining rats, following the administration of large amounts of magnesium sulphate. The rats were given no food for a period of twenty-four hours and then fed bread soaked with a saturated solution of Epsom salt. The rats which were negative for intestinal amœba when examined on three consecutive days, by this method were also negative at autopsy when the intestine and cecum were carefully examined for the parasites. This led the writer to feel that he had a comparatively safe method of detection of rats negative for amœbæ but not for curing rats of amœbic infection, as is incorrectly stated by Wenyon (1926) and Knowles (1928). The method, however, has not been thoroughly tested for the detection of the intestinal flagellates of rats and should be repeated with this objective in view.

Chiang (1925) felt that giving an enema by means of a small French catheter was a better method to employ. The writer has used this method but because of the fact that the cecal valve of the rat prevents the entrance of a catheter into the cecum and thus makes it impossible to collect unemptied cecal material by the enema, the writer feels that the use of a purgative which enters the cecum and insures passage of cecal contents into the colon is a more satisfactory method than the one proposed by Chiang.

The necessity of repeated examination of animals employing a combination of the above-mentioned methods is emphasized, and building up a stock of negative animals is recommended.

2. KITTENS

In addition to *Isospora,* which is often associated with definite diarrheic symptoms in kittens, natural infections of three other intestinal protozoa have been reported. They are an amœba resembling *Endamœba histolytica* of man and producing a similar acute infection. One case has been reported by Franchini (1920) in Italy and three cases of this natural amœbiasis were observed by the writer in Peking (Kessel, 1928*b*).

The methods for carrying on experimental amœbiasis in kittens will be discussed elsewhere in this monograph by Dr. Rees, so it is unnecessary to elaborate them in this chapter. However, the writer feels that in all experimental work with kittens, whether for amœbiasis experiments or for infection experiments with flagellates, the kittens should be subjected to the following three methods for the detection of natural infections:

I. The feces should be examined daily according to ordinary laboratory routine, employing both the preliminary Donaldson's stain and examination of the permanent smear stained with iron hæmatoxylin for a period of not less than two weeks.

II. On three successive days during the two weeks, kittens should be given a drastic purge of magnesium sulphate and the feces examined as described above and cultured in the L.E.S. medium of Boeck and Drbohlav (1925) or its modifications. The Epsom salt method is especially essential for the detection of natural *Giardia* infection.

III. Three high colon enemas should be given at intervals during the two weeks and the material collected by the catheter during the enema should be subjected both to microscopic examination and to culture. This method is specially important in detecting natural infections of *Trichomonas* and amœbæ.

Trichomonas

Trichomonas was first reported by da Cunha and Muniz (1922) in South America, who named the organism *Trichomonas felis.*

They found this in one kitten only, and could not distinguish it morphologically from the common *Trichomonas hominis* of man.

Other reports from Brumpt (1925) and Tanabe (1926), and Kessel (1926*a* and 1928*c*) record finding natural infections of *Trichomonas* in kittens.

Giardia

Giardia has often been reported from cats, having first been seen by Grassi (1881). Deschiens (1925) gave the name *Giardia cati* to the form which he found in the cat. Hegner (1925) gave the name *Giardia felis* to the parasite which he found in the cat in America, basing his species difference on the length to breadth ratio which he considers makes this organism different from *Giardia* of the dog and of man. Experimental attempts, thus far reported, to infect kittens with human *Giardia* have been meager. Fantham (1917) reports successful results and Deschiens (1921) also claims to have infected two cats with *Giardia* from man. Wenyon (1926), however, records three negative attempts to infect kittens with *Giardia lamblia* of man.

The writer has this problem under investigation at present and thus far has been successful in establishing human *Giardia* in six of eleven kittens. In one series of five kittens the first attempt from the first human case was negative, the kittens being examined at intervals during a period of six weeks following the attempt. Material from Case 2, however, yielded positive results and *Giardia* became established in four of the kittens, three of the kittens being definitely shown to pass cysts of *Giardia*. The experimental infection remained established in two of the animals for a period of three months, one of the others having been sacrificed early in the experiment and the fourth having met an accidental death.

The writer has not attempted the use of a duodenal tube in detecting *Giardia* in kittens. It would probably be useful if one were sure that the *Giardia* infection were restricted to the duodenum. However, this is not always the case, for the writer has found natural infections of *Giardia* in the jejunum and ileum as well as the duodenum of cats. In the current experiments with *Giardia* in kittens the writer is adopting the policy of rearing the kittens in the laboratory, and insuring a negative litter by sacrificing the mother. While kittens may be naturally infected by feeding the organisms mixed with their food, yet it is relatively easy to give a forced feeding by means of a stomach tube, as described earlier in this chapter.

Infection by rectum has been accomplished in experimental amœbiasis and trichomoniasis and the use of the rectal plug is recommended by some as enhancing the possibility of infection by producing a stasis of the bowel. The dangers, however, of intestinal obstruction and of producing secondary infection accompanying the removal of the celloidin plug offset to a considerable degree the advantage of a possible higher incidence resulting from its use. The writer recommends that it be avoided wherever possible. If the attempt to establish the infection is by the rectal method, the kitten should be prepared previously by fasting for eighteen hours; occasionally it is advisable to administer an enema prior to giving the infected material by rectum. The Luer syringe and French catheter are indispensable in infection work with kittens.

3. DOGS

Endamœba

Spontaneous amœbic dysentery has been reported from the dog by Kartulis (1891 and 1913) in Egypt, Darling (1915) in Panama, Ware (1916) in India, Fischer (1918) in China and Bauche and Motais (1920) in Cochin-China. Darling (1915) proposed the name *E. venaticum* for the amœba producing canine dysentery but Wenyon (1926) favors the assumption that the dysenteric amœba found in dogs is the same as *E. histolytica* of man.

Trichomonas

Trichomonas has been recorded from the dog by Brumpt (1925) and by Chatterjee, Harendranath and Mitra (1927) from the jackal. Brumpt (1925) considers his *Trichomonas* of the dog to be the same as the common trichomonad found in the domestic cat. The form found in India by Chatterjee was a pentaflagellate form and was named *P. canis-auri*. The species seen by the writer in Peking was also a *Pentatrichomonas* form and answered more to the description of Chatterjee's form than to the common *Trichomonas* seen by the writer in man and in kittens.

No experimental infection work has been reported thus far in dogs with *Trichomonas hominis*. In a series in progress by the writer one of four puppies experimentally infected has acquired the infection.

Attempts also should be made to infect dogs with the *Trichomonas* of rats, pigs, monkeys and cats.

Giardia

Giardia has been reported from dogs quite frequently, having first been noted by Grassi (1881). Hegner (1922) states that it possesses a characteristically broad anterior end and proposes the name *Giardia canis*. Animal-infection experiments seem to have been neglected with the dog, the only results recorded being those of Howitt mentioned by Thomson (1926) in which dogs were reported to have been successfully infected with *Giardia* from human feces.

Simon (1922) and Hegner (1922) have proposed a biometric method for differentiating species of *Giardia* on the basis of their length to breadth ratio. Since, however, the races of *Giardia* encountered in man exhibit such a wide variation it seems to the writer (Kessel, 1928*d*) that many more human cases must be considered as well as more cases of *Giardia* from the species of animals in question before it will be possible to make any definite statements on this point. The author has seen individual races of *Giardia* from man that correspond in their measurements and biometric ratios to the *Giardiæ* reported from rats, mice, cats, dogs, and monkeys. A single case, or even two or three cases from the cat, the dog, the monkey or any other animal are quite inadequate as the basis for establishing a new species, since the races of *Giardia* found in man display such a wide variation in size and shape.

4. THE DOMESTIC PIG

The intestinal protozoa of the domestic pig afford an important problem for investigation from the point of view of public health. They are also of interest theoretically because so many of them resemble the protozoa of man. Prowazek (1912) has described an amœba of the pig which he calls *Endamœba polecki*. Smith (1910) described an *Endamœba* of the pig which exhibited ability to penetrate the tissue of the intestine of pigs infected with hog cholera. No cysts of the amœba were described with certainty. Nieschulz (1924) gives the name *E. debliecki* to an amœba which he found in pigs. From his account it appears to the writer that he was merely dealing with a small race of the common *Endamœba polecki*. Accounts in the literature of the common amœba of the pig generally agree that the encysted stages produce only a single nucleus. Whether Smith (1910) was dealing with this amœba or with a different species is not quite certain, but on account of the fact that Smith's amœba penetrated the tissue the writer is inclined to feel that he was not dealing with the amœba described by Prowazek. Douwes (1921) figures a four-nucleate cyst of the amœba from the pig and Kessel (1928*a*) found an amœba resembling *E. histolytica* to be fairly common in pigs in Peking. Morphologically, the trophozoites and cysts are indistinguishable from *Endamœba histolytica.* The amœba was experimentally transferred to kittens which developed symptoms identical with those kittens experimentally infected with *Endamœba histolytica* of man. Young pigs reared under laboratory conditions were then experimentally infected with *Endamœba histolytica* of man by feeding them cysts. The infection became established and four-nucleate cysts were recovered from the pigs at intervals for several weeks. However, without a satisfactory explanation both naturally and experimentally infected pigs cleared themselves of the infection. Whether there is a slight physiological difference between the *Endamœba histolytica* of man and its cotype in the pig is not thoroughly demonstrated but it seems plausible to assume that the pig may be a temporary and not a permanent host for *Endamœba histolytica.* Further experimental work is needed on this subject to afford final conclusions. Other intestinal protozoa found in the pig are *Iodamœba suis* O'Connor (1920), *Trichomonas suis* Gruby and Delafond (1843); types resembling *Endolimax, Endamœba coli, Chilomastix* and *Bodo* are described by Kessel (1928*a*). *Balantidium coli* has also long been recognized as the common parasite of the domestic pig. Epidemiological and experimental evidence collected to date by Walker (1913) and Brumpt (1909) supports the hypothesis that *Balantidium coli* of man and of the domestic pig are in reality the same species. Since the other kind of intestinal protozoa of the domestic pig with the exception of *Endamœba polecki* resemble so closely in morphology a corresponding type found in man, the writer is inclined to acknowledge them as belonging to the same species as their representative types in man.

It must be remembered, however, that cross-infection experiments with these protozoa of the pig to common laboratory animals have not been done. Such are highly desirable and also other attempts to infect pigs with the common protozoa of man should be made. Kessel (1928*a*) reports having infected young pigs with *E. nana*, *E. coli*, and *Chilomastix*, and we generally assume the *Iodamœba* of the pig and man are identical. However, no actual experimental work has been performed in which the *Iodamœba* of man has been experimentally transferred to the pig. The chief difficulty that the writer has experienced thus far has been that he has never been able to find, without doubt, a young animal which has been free from a natural *Iodamœba* infection. Apart from Smith's (1910) observation of an amœba in the tissue none of the other common intestinal protozoa have been found to produce lesions in the pig.

The writer hoped that the pig might be found to be a suitable animal for protozoiasis studies, but it appears, for the most part, at least, that the pig serves merely as a temporary reservoir host without exhibiting pathological symptoms. Further, on account of their size pigs are not animals to be kept easily under laboratory conditions. The extreme length of the large intestine which is filled with a large amount of fecal débris makes them difficult animals with which to work. Routine examination of normally passed feces with additional examination following a purge are the only practical means that can be employed for detecting infections in pigs, since the cecum is so large that it is not practical to consider collecting fecal material by enema.

5. RABBITS

The rabbit harbors at least three intestinal protozoa similar to ones found in man. They are *Chilomastix cuniculi* Fonseca, 1915, *Giardia duodenalis* Davaine (1875) reported by Schwartz and Shook (1929) to be pathogenic and the cause of ulcers, and an amœba of the *E. coli* type seen by Brug (1918) in rabbits and by Rudovsky (1923) in hares. The writer also has observed this amœba recently in rabbits in Southern California. Little animal transmission work has been attempted with rabbits but Huber (1909) and Thomson (1926) report having established *E. histolytica* infection in rabbits.

There is a great need for careful morphological study and animal-transfer experiments using the protozoa of the rabbit, since so little has been accomplished to date in this field. The COCCIDIA of rabbits have attracted much more attention because of their association with liver and intestinal coccidiosis. It is surprising in this connection, however, how much careless work has been done in the line of species differentiation. Even in the most recent literature on the subject Perard (1925) and Waworuntu (1924) the liver and intestinal types are not clearly differentiated. Recent studies in the writer's laboratory on this subject have led to the differentiation of five species of COCCIDIA in rabbits, all *Eimeria,* one being a liver type and four being intestinal types. With the exception of one species, the intestinal COCCIDIA may be differentiated from *E. stiedæ* of the liver by the presence of a residual body outside the sporocysts in the ripe oöcyst. The fourth intestinal type simulates rather closely the liver type and is differentiated from the same by size and shape of the micropyle. The five species of *Eimeria* in rabbits recognized in recent studies are:

E. stiedæ Lindemann, 1865, the common liver type. Shows no residual body in ripe oöcyst.

E. magna Perard, 1925, large intestinal type with pronounced lips bounding micropyle. Prominent residual body present.

E. perforans Leuckart, 1879, small intestinal type without noticeable lips or micropyle. Residual body present.

E. media sp. novo., smaller than *E. magna* and without pronounced lips bounding micropyle. Residual body present.

E. irresidua sp. novo., large intestinal form without residual body. Lips bounding micropyle less pronounced than in *E. magna.*

Host-parasite relationships between the COCCIDIA of rabbits and of other animals, particularly guinea-pigs, rats, and other closely related forms, need to be investigated in greater detail than observed heretofore, although studies to date (Andrews, 1927, and Wenyon, 1926) indicate, with the exception of the *Isospora* of cats and dogs, that the specificity of host-parasite relations among COCCIDIA are quite rigid.

6. GUINEA-PIG

The guinea-pig, though a common laboratory animal, harboring many intestinal protozoa, has never been used extensively in animal-transfer experiments. *Endamœba cobayæ,* Walker, 1908, a form resembling *E. coli,* has been seen by Chatton (1918), Wenyon (1926), and Holmes (1923). Leger (1918) and Baetjer and Sellards (1914) and Chatton (1918) have also recorded a tetranucleate type. Hegner (1926) names *Endolimax caviæ* from the guinea-pig, and also *Giardia caviæ* (1923). *Trichomonas caviæ*

Davaine, 1875, is very similar to *T. muris*, exhibiting the same coarse structure and it forms cysts which are also formed by *T. muris*. Experiments should be designed to determine whether or not these are cross-infective. *Chilomastix intestinalis* Kuczynski, 1914, has also been described, and *Embadomonas* has been observed by Wenyon (1926). The common coccidium of the guinea-pig observed by the writer resembles more closely an *Eimeria* found in the rat than any in rabbits. This is probably the one named *E. caviæ* by Sheather in 1924. The *Balantidium* of the guinea-pig has been named *B. caviæ* by Neiva, da Cunha and Travassos (1914). Whether all the above forms can be accepted as representing distinct species will depend on further accurate morphological differentiation and on transfer experiments. These appear to be of immediate interest especially with rabbits and rats.

7. AVES

Hegner's (1928*d*) recent experiments with chicks in which he has established experimental infections of protozoa from several different mammals in young negative chicks opens up an exceptionally interesting and valuable field for investigation in the study of relationships between the intestinal protozoa of birds and mammals. Of immediate practical importance is the possibility of the fowl's serving as a reservoir host for human flagellate infections. Another problem of special importance is cross-infection with intestinal COCCIDIA between domestic rabbits and domestic fowls, both often being raised in close proximity on the same farm and both suffering from severe intestinal coccidiosis. A preliminary report of work being carried on in the writer's laboratory on this problem indicates that chicks at least are not readily infected with rabbit COCCIDIA, for twelve chicks fed ripe oöcysts from all four species of intestinal COCCIDIA of rabbits have failed to acquire the infection.

CHAPTER XI

EFFECTS OF CHANGES IN THE DIET OF THE HOST ON INTESTINAL PROTOZOA

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INTRODUCTION

The purpose of experiments on diets and intestinal protozoa is to determine something of the relation of an animal's food to its susceptibility to infection with protozoan parasites. If mammals are divided into three general groups according to food habits—(1) meat eaters, (2) vegetable eaters, and (3) an intermediate group that eats both meat and vegetables—it will be found, upon consulting a comprehensive check list, that PROTOZOA have been reported rarely from the first group; that strictly herbivorous animals have a distinct and specialized protozoan fauna, while the omnivorous animals are parasitized by more general types of PROTOZOA, some of which occur in both of the former groups. The majority of animals do not, however, lend themselves to definite experimentation which must be conducted under controlled conditions in the laboratory. There are several primary considerations which must be taken into account in planning studies upon the relation of diet to infections with intestinal protozoa. In the opinion of the writer these are (1) methods for determining the species and numbers of protozoa present in the intestine of an animal, and the conditions under which these organisms live, (2) diets and experimental animals, and (3) intestinal protozoa of experimental animals.

METHODS

1. *Intestinal Protozoa—Numbers and Conditions in Intestine.* Fecal examination over a period of several days is usually sufficient to determine the species of PROTOZOA present in animal's

digestive tract. Dobell (1917), referring to infection in man, states that "the expectation for the average infected case is that it will be found positive twice in every five examinations." This method is, however, time-consuming and, with such animals as rats, which pass solid feces, is unreliable. Brug (1919) states that "cysts (of amœbæ in rats) may be found for several days in succession, then for several days, weeks or months nothing may be found." This difficulty with rats may be overcome by starving them for several hours, then feeding bread that has been soaked in a saturated solution of magnesium sulphate (Kessel, 1923). Rats react to the purgative-passing semi-liquid feces in about twelve hours. If the animals are infected one can usually find abundant protozoa in material passed after such treatment. Kessel claims to be able to detect practically all cases of amœbic infections in rats after two examinations by this method.

Chiang (1925) advocates the use of a "high enema" of sodium chloride solution as a substitute for examinations of rats by purgative. He has employed this method in examinations for amœbæ, and claims it is just as effective as purging and much more rapid. The method is as follows: A syringe is filled with 0.85% sodium chloride solution and attached to a small rubber catheter which is lubricated with oil or vaseline. The catheter is inserted ten to fifteen centimeters into the rectum and five to ten cubic centimeters of salt solution injected into the colon and cecum. The rat retains the enema for several minutes. When passed it is collected in a large Petri dish, and examined for protozoa. Ether anesthesia may or may not be used, depending upon the temperament of the rat.

These methods, though effective in determining the presence of protozoa in the intestine of animals, are not suited for accurate enumerations of the organisms actually living in the intestine. They either depend upon finding organisms naturally passed in the feces or carried out under abnormal conditions. Purgatives may, under certain circumstances, entirely eliminate infections. Kessel (1924) states that seven of nineteen control rats became negative for amœbæ following examination by the magnesium sulphate method. Such an occurrence would no doubt lead to erroneous interpretations of results if diets were being tested. Hence these methods cannot be recommended.

Surgical methods that enable one to remove a measured amount of material from the part of the intestine inhabited by protozoa have been successfully employed in studies on the effects of changes in diet on the trichomonads of rats. Such methods seem almost essential in investigations of this sort. They allow observations on the protozoa as they live in the intestine with minimum disturbance to intestinal conditions. They are also applicable to the study of other protozoa of the rats and, with modifications, to similar studies on the intestinal protozoa of other laboratory animals. In the experiments on the trichomonads of rats 0.2 of a cubic centimeter of material was removed from the cecum, and diluted in 0.8 of a cubic centimeter of 0.85% sodium chloride solution, care being taken to insure thorough mixing and breaking up of all masses of material. From this dilution one cubic centimeter was transferred to nine cubic centimeters of saline to serve as a base for further dilution in typing and enumerating the bacteria. The remaining cubic centimeter was used for trichomonad counts and pH determinations. The flagellates were counted in a Leitz blood-counting chamber, the organisms in the four groups of large squares being counted and averaged. This was taken as the number of protozoa per 0.1 cubic millimeter of a 1:10 dilution. This figure multiplied by one hundred was taken as the number of trichomonads per cubic millimeter of cæcal material. The percentage of error is considerably less than ten per cent when infections vary between 1,000 and 40,000 trichomonads per cubic millimeter. Heavier or lighter infections are rarely encountered.

The pH was determined colorimetrically using a LaMotte 36 hydrogen-ion testing set. This method is not as accurate as electrometric determinations, but is applicable when an electric apparatus is not available. These measurements are of doubtful value, however, considering what is known of the pH of media in which intestinal protozoa are cultivated.

The bacteria that should be considered are those types which are known to respond to changes in diet, *i.e.*, the lactose-fermenting organisms, the aciduric bacteria and the anaërobes. All protozoa that live in the lower intestine are probably influenced to some degree by changes in bacteria due to dietary alterations. Those that ingest bacteria, *Trichomonas, Chilomastix,* and *Endamœba,* no doubt are more readily influenced, but certain bacteria probably exert more influence through the substances they form from partly digested food than through the numbers present.

In the studies on trichomonads, an attempt was made to enumerate (1) the lactose-fermenting organisms, (2) the aciduric bacteria and (3) the anaërobic bacteria. Lactose-litmus-agar was employed in plate counts of the first group, and tomato juice-peptone agar (Kulp, 1927) in plate counts of the second group. Dilution counts were made of the anaërobes in cooked meat medium (Kahn, 1922). The intestinal material was diluted to an extent found to be appropriate and one cubic centimeter amounts planted in these media. Kendall's *Bacteriology and A Treatise on the Transformation of the Intestinal Flora* (Rettger and Cheplin), 1921) will be helpful in understanding the bacteria involved. Ford's *Text-book of Bacteriology* describes the preparation of media.

It seems essential in understanding the influence of food on the intestinal protozoa of any animal that similar observations be made.

2. *Diets and Experimental Animals.* Diets must contain all substances necessary for growth and reproduction of experimental animals, and all ingredients should be thoroughly mixed. Laboratory rats are well standardized, and it is possible to alter their food so that their intestinal conditions may be made to simulate those of other animals. The writer (Ratcliffe, 1928) published a list of diets, planned by the Department of Chemical Hygiene of this school, that are known to produce a variety of conditions in the intestine of rats. It is probable that these diets could be used with mice or guinea-pigs, but experiments would be necessary to determine this.

Diets for other laboratory animals, with the exception of the domestic fowl, have not been standardized satisfactorily to allow experimental observations comparable to those possible with rats. With chicks it is possible to procure satisfactory food mixtures in a dry form, but the degree of modification possible with the diets has not been determined so far as is known to the writer. Neither is it possible to remove samples from the intestine of chicks by surgical methods. However, Dr. Robert Hegner has found that intestinal protozoa of chicks live in the ceca, and that material is passed from this portion of the intestine separately and is different in color from material passed from the colon. This allows one to obtain material closely approximating that in which the protozoa live. Hence it is possible to make observations on intestinal protozoa of chicks similar to those on rats, provided diets can be planned successfully.

With the exception of laboratory rats and chicks other laboratory animals must be standardized before it will be possible to employ them in anything but the crudest type of experiments.

PROBLEMS

1. *Natural Infections.* By natural infection is meant infection with intestinal protozoa that is acquired by the young from the mother.

a. The relation of all intestinal protozoa of rats or fowls to changes in diets may be studied by the methods described above.

b. A more detailed study of changes in intestinal flora accompanying changes in diet may lead to a better understanding of the problem and determine the effects of bacteria due to numbers as well as effects due to metabolic products.

2. *Cross-infection.* Parasite-free animals must first be obtained before work of this nature can be attempted. Chicks, of course, are readily available, while a breeding stock of parasite-free rats may be established by the following method.

Since young rats become infected with intestinal protozoa from the mother while they are being weaned and are beginning to eat solid food, young may be taken from the mother soon after the eyes are open, fifteen to twenty days after birth, and reared to maturity free from intestinal protozoa, provided food is not allowed to become contaminated and contact with infected animals is prevented.

The chief difficulty will be encountered in inducing the young animals to eat. Whole milk powder dissolved in warm water may be fed for the first day or two. It is usually necessary at first to give the milk to each animal separately by means of a pipette. Dry food, green vegetables and water may be given after the second day, the food and water being placed in shallow vessels in the cage.

About two-thirds of the young may be expected to survive this treatment, but once a breeding stock of parasite-free rats has been established animals can be reared in the usual way.

The following problems are suggested:

a. Cross-infection experiments will allow a study of the relation of diet to specificity of parasites.

b. One may also investigate the relation of diet to differences in protozoan fauna of animals that are closely associated.

c. Identity of species of PROTOZOA occurring in different animals may also be investigated by these methods.

CHAPTER XII

THE ROLE OF BACTERIA IN THE CULTIVATION OF INTESTINAL PROTOZOA

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To date no intestinal protozoön from mammals has been grown in culture except in the presence of bacteria. It has been known for some time that many species of intestinal protozoa ingest bacteria in the intestine of the host and in cultures, but, until recently, nothing was known as to the importance of bacteria in cultures of protozoa or the relative suitability of various types of intestinal bacteria as food for intestinal protozoa. It was obviously impossible to determine the importance of bacteria in cultures or the relative value of different species without first separating the protozoa from the bacteria.

Attempts have been made to free protozoa of bacteria but, with the exception of *Tritrichomonas fecàlis* Cleveland, all attempts have failed. It seems probable, with the technique employed by Cleveland (1928), that other species will also be separated from bacteria, and similar studies made on other organisms. Dr. Cleveland's methods are quoted.

"It is obvious that, in order to free trichomonads of bacteria, it is necessary, first of all, to develop a culture medium which is at least as well-suited to the growth of the trichomonads as to the growth of the bacteria. Most media in which trichomonads have been cultivated are much better suited to the growth of the bacteria. Consequently, an effort was made to develop a medium (or to find a condition) as well-suited to the growth of the trichomonads as the bacteria. Many experiments were carried out which, owing to the fact that they were negative, that is, did not yield the desired result, will not be mentioned. Various chemicals were used with the hope that some of them would inhibit the growth of the bacteria more than that of the trichomonads, but, as Andrews (1926) states, this appears to be almost a hopeless undertaking. Certainly most of the substances used inhibited the growth of the trichomonads far more than the bacteria. All there was left to do then was to improve the methods of cultivating trichomonads, so that they might be grown more abundantly.

"After experimentations with many types of media, it was found that a medium composed of about one gram of Loeffler's dehydrated beef serum to 100 cc. of 0.7 per cent sodium chloride gives a fairly abundant growth of trichomonads. Buffering of the medium sometimes increases the growth, depending on the bacteria that are dominant. This medium was made in great quantities and was run through the large Berkefeld filters (V, No. 1). It was then run through another Berkefeld filter in a tubing and sterilizing apparatus, which is illustrated in Fig. 11. This apparatus was wrapped with paper and sterilized in the autoclave each time it was used. It was possible to sterilize the medium in this way without heating it and to fill tubes at the rate of two to three hundred an hour. After being filled, the tubes were incubated for four days at 36° C. to test their sterility. Far less than one per cent of the tubes filled were contaminated in the filling process. Four and five hundred tubes have been filled many times without a single tube being contaminated. Hundreds of liter flasks of the medium were also filled and sterilized in the same manner as the tubes.

". . . Inoculations were made from tubes in which the organism was growing abundantly to liter flasks of serum-saline medium (prepared and sterilized as described above). The organism grew very well in this large quantity of medium. The growth was more abundant, however, when the flasks were filled so that the fluid came up into the neck of the flask. The organism grew so well that from one to two cubic centimeters of *Tritrichomonas*, having the appearance of heavy cream, could be obtained when the organisms in a liter were concentrated by centrifugalization. It was discovered later, after a number of the washing experiments, to be described presently were carried out, that the bacterial growth could be cut down greatly and without any decrease in the number of trichomonads, by placing vaseline on the surface of the fluid in the neck of the flasks. The lack of oxygen cut down the growth of many types of bacteria, but did not affect the rate of multiplication of the trichomonads.

"Now that a method had been developed for growing the trichomonads in great quantities and without the tremendous overgrowth of bacteria that usually occurs, the possibility of freeing the organism of bacteria seemed more promising. Many physical processes were employed, but most of them met with little or no success and will not be described here. In the earlier experiments the organism was washed in sterile sodium chloride in concentrations varying from 0.5 to 1 per cent. After the trichomonads had been washed in saline three or four times, they were suspended on the surface of sterile saline in an upright tube ten feet long. The organisms, being larger and heavier than bacteria, would fall downward through the saline faster than the bacteria, and it was possible, in a few instances, to collect drops of saline from the bottom of this tube which contained from one to three trichomonads each and no bacteria. Twenty drops were taken in succession by opening the stopcock at the end of the tube (the lower end of the tube was well protected from contamination of air microörganisms by a tube twice its size fitting over it and extending beyond it. The large tube was plugged with cotton. The entire apparatus was sterilized in the autoclave). Ten of these drops (every other one) were placed on glass slides and examined microscopically for trichomonads, and ten were placed in sterile serum-saline medium. Some of these drops contained no trichomonads, while others contained from one to three organisms each. About half the tubes inoculated in this manner showed no bacterial growth after ten days. Approximately two hundred tubes were inoculated in this way, but *Tritrichomonas* did not grow in any of them unless bacteria were present. These results indicated that *Tritrichomonas* was not able to grow without bacteria—at least not in any of the

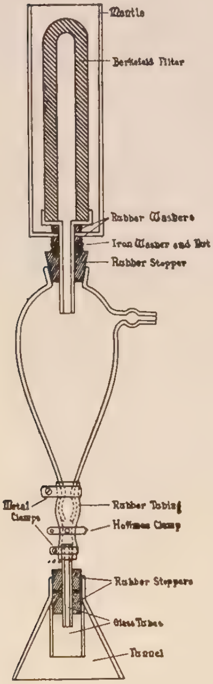

FIG. 11.—Apparatus for sterilizing and tubing media. (After Cleveland, 1928.)

media that were tried—but the fact was not demonstrated clearly. . . . It was impossible to wash the organisms more than four to five times in saline, because they would round up, become non-motile, and die. However, after almost giving up the undertaking entirely, I discovered that the trichomonads could be washed as many times as desired provided sterile serum-saline medium was used instead of saline. It then became necessary to make several gallons of serum-saline medium and to sterilize it by filtration. Two hundred liters of this medium were made, sterilized, and stored away in the cold room. Then the endeavor to free *Tritrichomonas* from bacteria really began.

"In each attempt to free the trichomonads of bacteria the following procedure was carried out: the organism was grown in liter flasks which were examined from time to time to note the abundance of growth. As soon as the trichomonads reached their maximum number in the culture, ten liters were run through filter paper three or four times to remove as many as possible of the particles of débris larger than the trichomonads. Then the *Tritrichomonas* of the ten liters was concentrated by centrifugalization. This gave ten to fifteen cubic centimeters of *Tritrichomonas* having the appearance of heavy cream. Then sixteen sterile fifty cubic centimeters pointed centrifuge tubes were filled with sterile serum-saline medium near the freezing point of the medium and the *Tritrichomonas* obtained from the ten liters was layered on the surface of the fluid in the centrifuge tubes. The centrifuge (International size 2) was run at step six of the rheostat for fifteen minutes. One-half to one cubic centimeter of *Tritrichomonas* was packed in the bottom of each tube after centrifugalization. Practically all the fluid was removed from each tube with sterile pipettes. Then the *Tritrichomonas* was removed from each of the tubes and layered on the surface of the fluid in sixteen other centrifuge tubes. The centrifuge was run as before. Sterile serum-saline medium near the freezing point of the medium, sterile pipettes, and sterile tubes were used in every step of the washing. In the last three or four washings the tubes were plugged with sterile cotton plugs which were held in place by a rubber band around the outside of each tube. After this washing process had been repeated twenty times, the ratio of *Tritrichomonas* to bacteria was about fifty to one. This was determined by dilution counts of the number of trichomonads in twenty loops (two millimeters fused, round, platinum-iridium, which delivered approximately the same amount of fluid each time) of the *Tritrichomonas* after washing. The number of bacteria present in each of twenty similar loops was determined by plating on solid media.

"The ratio of bacteria to *Tritrichomonas* was so small now that it was an easy matter to inoculate tubes containing from ten to fifteen trichomonads each and no bacteria. This, of course, was done by diluting the trichomonads in sterile serum-saline medium. After the completion of most of the washing experiments, *Tritrichomonas* was so abundant that when a two millimeter loop of the organisms was placed in 100 cubic centimeters of serum-saline medium, a two millimeter loop of this dilution would contain fifteen to twenty trichomonads. This was determined by counting twenty loops. Inoculations were made then from this dilution of *Tritrichomonas* to tubes of various sterile media. It was usually found most convenient to make the inoculations when the fluid was in a large Petri plate. From seventy to ninety per cent of the tubes inoculated in this manner showed no growth of bacteria. It was later found that even a higher percentage of bacteria-free tubes could be obtained by securely fastening the bottom of a large Petri plate to a steady table, covering the surface of the plate with sterile serum-saline medium, and then placing a loop of the washed trichomonads in the centre of the plate, care being taken to stir or agitate the fluid as little as possible. After various intervals, loops from different distances from the centre were picked up and examined microscopically for trichomonads. The trichomonads would swim away from the centre faster than the bacteria. When ten to fifteen trichomonads were found to be present in a loop taken at a certain distance from the centre (usually two to three inches), tubes of sterile serum-saline and other media were inoculated from this place. It was also possible by this method to obtain bacteria-free trichomonads with less washing than when a loop of the washed trichomonads was diluted and thoroughly shaken in serum-saline medium. But the distribution of the trichomonads in the dilution that was not shaken was, of course, not as uniform, and it was impossible to be certain as to the number picked up with a loop. It should also be stated that in the earlier washing experiments, the percentage of bacteria-free tubes was much lower. It was only after the washing procedure was fairly well perfected that the number of bacteria could be reduced so low. In other words, in the earlier washing experiments the trichomonads were not washed so free of bacteria. Only three or four out of a hundred tubes inoculated would fail to show bacterial growth. . . . Now . . . it was possible to be certain that inocula containing from ten to fifteen trichomonads each could be placed in one hundred tubes, seventy to ninety of which contained no bacteria."

After this tedious and time-consuming method was perfected, attempts were made to grow the trichomonads in pure suspensions of heat-killed bacteria in the serum-saline medium and in many other media. The protozoa failed to grow in any of these media, but a chance contamination of one tube by an obligate psychrophylic bacterium made it possible for Dr. Cleveland to test trichomonads at 36° C. with living bacteria instead of those killed by heat, and still be sure he was dealing with pure cultures. *Tritrichomonas* grew exceedingly well with this psychrophylic organism at room temperature, but this bacterium would not grow at a temperature above 34° C. while the trichomonads grew well at room temperature or at 36° C. All that was necessary, therefore, was to inoculate sterile media with trichomonads from the stock culture and with the bacterial organism to be tested and incubate at 36° C. The psychrophylic organisms from the stock culture died in four to five days and the trichomonads were then in a pure culture of bacteria growing at 36° C.

PROBLEMS AND SUGGESTIONS

This technique may be applicable to similar studies on other species of trichomonads, if cultures can be induced to grow at temperatures lower than 37.5° C. The availability of obligate psychrophylic bacteria is not known to the writer. Cleveland (1928) states that only three or four species are known.

Methods for studying other species of intestinal protozoa can only be suggested since they have not been experimentally tested. *Chilomastix,* an intestinal flagellate that forms cysts, may be grown in a medium containing one part human blood serum and four parts Locke's solution (Boeck, 1921). Locke's solution may be autoclaved and the serum filtered to insure sterility or the medium may be mixed and filtered as described for *Trichomonas* medium. Certain species of *Chilomastix* may also be satisfactorily grown on the *Trichomonas* medium. Cleveland's technique for establishing *Trichomonas* in pure culture of bacteria may also be applicable to this protozoön, if it is possible to maintain *Chilomastix* at room temperature.

When cysts are available they could be washed free of bacteria or possibly treated with a disinfectant or acid solution to free them of bacteria. If this proved possible, the cysts of *Chilomastix* could then be hatched at 37.5° C. (Hegner, 1927) in media that had been previously seeded with a single species of bacterium. This is only a suggestion. It might also be possible to induce encystment in cultures (Cunha, 1927) as has been done with *Endamœba histolytica.*

The cultivation of *Endamœba histolytica* (Chap. XIX) will not be discussed here, but it may be suggested that cysts are readily produced in cultures and may be freed from bacteria by treating with N/20 hydrochloric acid and washing in sterile distilled water (Dobell, 1928). It would, therefore, be easily possible to test the suitability of various bacteria as food for these organisms and to determine their influence upon cultivation of the amœbæ.

Balantidium coli cysts have been cultivated by several investigators (Chap. XXIV). If cysts were available, it might be possible to induce these organisms to develop in pure cultures of bacteria. It might also be possible to free the trophozoites from bacteria, by methods already described.

The solution of the problems suggested above would no doubt provide much information regarding the interrelations of intestinal protozoa and bacteria, the relations of an animal's food to the specificity of protozoan parasites and the identity or individuality of species found in different animals.

CHAPTER XIII

INTESTINAL FLAGELLATES IN GENERAL

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The MASTIGOPHORA comprise a class of PROTOZOA containing large numbers of both free-living and parasitic species. It is convenient to separate the parasitic species into two groups, intestinal flagellates and blood-inhabiting flagellates. The latter belong almost entirely to the family TRYPANOSOMIDÆ. In the following chapters problems and methods of research are discussed with respect to certain genera of intestinal flagellates and certain groups that occur in several types of hosts. In this chapter a general survey of the intestinal flagellates will be made to indicate to what orders and families the more easily accessible species belong and in what animals one may expect to find them. The morphology and taxonomy of the MASTIGOPHORA are treated in Calkins' *Biology of the Protozoa*, pp. 248-297, and in Wenyon's *Protozoology*, pp. 268-312 and 607-715, and in Hegner and Taliaferro's *Human Protozoology*, pp. 201-265. Calkins also gives a key to genera on pp. 298-314.

The classification of the MASTIGOPHORA is a matter of opinion among students of the group. It is customary to divide the orders into two subclasses placing in the first subclass, to which the name PHYTOMASTIGINA is applied, several orders that contain plant-like flagellates and in the second subclass, the ZOOMASTIGINA, a group of four or five orders composed of animal-like flagellates.

Fewer parasitic species are present among the plant-like flagellates than among the animal-like flagellates. In the order EUGLENOIDIDA are a number of interesting species of plant-like forms including *Copromonas subtilis*, which is a coprozoic species about which much is known, *Astasia mobilis*, which is a parasite of the fresh-water crustacean *Cyclops,* and certain euglena-like forms that occur in the intestine of frog tadpoles and that have been described by Wenrich (1924) as *Euglenamorpha hegneri* and by Brumpt and Lavier (1924) as *Hegneria leptodactyli.* Probably other species occur in the intestine of tadpoles. That cross-infection experiments are possible was demonstrated in the case of *Euglenamorpha hegneri* by the writer (Hegner, 1923).

Another group of plant-like flagellates that contains a large number of parasitic species is the order DINOFLAGELLATA. Most of these belong to the family BLASTODINIDÆ which has been treated in detail by Chatton (1920), to whose work the reader is referred.

The animal-like flagellates are to be found in the digestive tract of almost every species of animal and each host as a rule contains many different species. Kofoid, for example, records the results of the examination of five species of AMPHIBIA, four species of reptiles and six species of mammals. Every species studied was found to be infected with intestinal flagellates and flagellates were obtained from all but two of the 329 individuals examined. Furthermore, each host species contained a number of species of flagellates. For example, the salamander *Diemyctylus torosus* was found to harbor fourteen species of flagellates belonging to ten genera. Probably only a small fraction of the intestinal flagellates in existence have been described and named. The order PROTOMONADIDA contains the majority of the parasitic MASTIGOPHORA, not only intestinal species but also blood-inhabiting species belonging to the family TRYPANOSOMIDÆ. Certain of the families that contain intestinal species will now be discussed.

In the family MONADIDÆ is the genus *Oikomonas* which contains not only a number of free-living species but also species that have been reported from the intestine of man and lower animals. The members of the genus are very small and on that account rather difficult to study. Resembling *Oikomonas* somewhat is the flagellate known as *Histomonas meleagris* which appears to invade the tissues of certain birds and bring about the disease known as blackhead in turkeys and fowls.

Among other flagellates belonging to the family MONADIDÆ that may probably be obtained without great difficulty and that would well repay study are species belonging to the genus *Rhizomastix* which have been reported from the intestine of AMPHIBIA and insect larvæ, and species of the genus *Heteromita,* among which is *Heteromita uncinata*, which has been observed in human feces. An interesting species, *Costia necatrix*, grows on the skin of fish to which it attaches itself by means of its flagella. It is accused of being pathogenic, especially to young fish reared in fish hatcheries.

In 1915, Fonseca proposed the genus name *Enteromonas* for a flagellate that he found in human feces in Brazil. The status of this genus and of the several species that have been described is not clear. It has been suggested that *Enteromonas* is the same genus as *Tricercomonas* which was found by Wenyon and O'Connor in 1917. Enteromonads have been described from rabbits and guinea-pigs and a careful study should be made of the intestinal flagellates of these animals in order to determine the validity of the genus *Enteromonas*.

A very interesting genus is *Tetramitus* to which a number of free-living species belong, some of which appear to have been found living in fecal material. Bunting (1926) has described a very remarkable life-cycle in a *Tetramitus* which she cultivated from the cæcal contents of rats. This life-cycle includes an amœboid stage, a flagellate stage and cysts. This work should be confirmed and extended to other intestinal flagellates that may have similar life-cycles.

A number of families of intestinal flagellates contain one or several species that have attracted the attention of investigators, most of which would repay further study. The family BODONIDÆ contains forms that have been reported from the feces and urine of man (Powell and Kohiyar, 1920). A species was also observed by Knowles and Das Gupta (1924) in saliva from the human mouth. *Bodo caudatus* frequently occurs as a coprozoic form and *Bodo edax* less frequently. The family PROWAZEKELLIDÆ includes an interesting species, *Prowazekella lacertæ*, which occurs in the intestine of lizards and salamanders. An interesting phase in its life-cycle has been described as cyst formation. Cysts containing about sixty-four flagellates occur in the intestine of the lizard. Transmission appears to take place when infected lizards are devoured by other lizards.

The next two families to be considered contain human intestinal flagellates. The family EMBADOMONADIDÆ includes *Embadomonas intestinalis* which was described from man by Wenyon and O'Connor (1917). Other species have been reported from lower animals such as the guinea-pig, sheep and sloth (Hegner and Schumaker, 1928). All of these species have been grown in culture in which they occur in large numbers. No doubt they are often overlooked in fecal material because of their minute size and small numbers. The genus *Embadomonas* was established for flagellates found by MacKinnon (1911) in the intestine of the larvæ of aquatic insects. No doubt many other species of embadomonads besides those already described are inhabitants of both vertebrates and invertebrates.

The human intestinal flagellate *Chilomastix mesnili* belongs to the family CHILOMASTIGIDÆ. The morphology of this species is not entirely clear, especially that of the cytostomal region. Species of the genus *Chilomastix* have been recorded from many of the lower animals but very little is known regarding their life-cycles, methods of transmission and relation to their hosts.

The family CERCOMONADIDÆ contains two interesting genera, *Cercomonas and Tricercomonas.* To the former belongs *C. longicauda* which is a common coprozoic flagellate in the feces of man and other animals. *Tricercomonas intestinalis* is a human intestinal flagellate which has been recorded from various parts of the world but does not seem to occur in a large percentage of individuals.

A number of interesting genera belong to the family TRICHOMONADIDÆ. Certain of these are discussed in Chapter XV. Trichomonads have been described from the human mouth, intestine, vagina, and urinary tract. Species most easily obtained for study are *Trichomonas muris,* that occurs in enormous numbers in the cecum of rats, and *T. augusta* of the frog. Many mammals, birds, cold-blooded vertebrates and invertebrates serve as hosts for species belonging to this genus. An interesting problem concerning the human trichomonads concerns the generic status of specimens possessing three, four and five flagella respectively. Those with three flagella are sometimes placed in the genus *Tritrichomonas,* those with five in the genus *Pentatrichomonas* and those with four are retained in the genus *Trichomonas*. Among the other genera that can probably be obtained easily for study are *Ditrichomonas* that occurs in termites, *Eutrichomastix* reported especially from reptiles, and *Polymastix* from the intestine of various insects and several other genera from termites.

The intestinal flagellates of termites are very numerous in species and complicated in morphology. They have been studied recently especially by Grassi (1917), Kofoid and Swezy (1919), Koidzumi (1921), Cleveland (1923, 1924, 1928), Kirby (1926) and others. They belong to the family DINENYMPHIDÆ and especially to the family TRICHONYMPHIDÆ of the order HYPERMASTIGIDA. To this order belongs also the flagellate *Lophomonas blattarum,* a common inhabitant in the intestine of the cockroach. Another family of termite flagellates, the CALONYMPHIDÆ, was founded by Grassi and is the only family in the order POLYMONADIDA.

Among the genera of particular interest in the order DIPLOMONADIDA are *Hexamita, Giardia,* and *Trepomonas.* The genus *Giardia* is discussed in Chapter XIV. *Hexamita* occurs both in fresh water and in the intestine of certain animals. *H. muris* is commonly present in the small intestine of rats and mice. A number of other species have been reported from birds, reptiles, amphibians, fish and insects. *Trepomonas agilis* is a peculiar flagellate that is coprozoic in habit and has been reported in human feces.

It is quite evident from a survey of intestinal flagellates that only a beginning has been made in the study of these organisms. Large numbers of species still undescribed are no doubt to be found in our commonest laboratory and domesticated animals. These species need to be described and named and their life-cycles worked out. Methods of transmission and host-parasite relations and host-parasite specificity would well repay study. The methods of procedure and the types of problems available may be inferred from the more detailed discussions of problems and methods in other chapters of this book.

CHAPTER XIV

INTESTINAL FLAGELLATES OF RATS

By

D. H. Wenrich

It is the purpose of this chapter to call attention to some of the known facts and some of the problems in relation to the intestinal flagellates of rats.

METHODS

1. *Obtaining fresh material*

A. *Feces.* Few flagellates are to be found in fully formed fecal pellets, except the cysts of *Chilomastix, Hexamitus* and *Giardia,* and these cysts are not numerous as a rule. When rats are handled, defecation is often stimulated so that soft feces become available. Soft feces may contain the trophozoites of flagellates which can be identified, but trichomonads from feces are usually not in good condition for study.

Laxatives and purgatives may be used. Hegner (1923*a*) fed sunflower seeds to bring down the flagellates and Kessel (1923) fed stale bread soaked in a saturated aqueous solution of magnesium sulphate as a purgative.

B. *Material from the cecum and small intestine* is usually obtained by killing the host, opening the abdominal cavity and removing the contents of the cecum and small intestine after making incisions in these organs. Killing with ether leaves the muscles of the rat in a relaxed condition and is preferable to killing with chloroform. Ratcliffe (1928) removed material from the cecum of rats under ether anesthesia.

2. *Examining fresh material*

A. *Examination of living flagellates.* Any of the following may be used for diluting cecal and intestinal contents for the study of living flagellates: physiological salt solution, Ringer's solution, Locke's solution and a solution made by adding 0.5 of a gram of sodium chloride and 0.5 of a gram of sodium citrate to 100 cubic centimeters of water. Any of these solutions is more satisfactory if diluted to about two-thirds the usual strength.

Intravitam staining of various cytoplasmic constituents may be accomplished by using Janus green B, 1-5000 in physiological salt solution (Hogue, 1926); Janus green, 1-1500 in 0.3% NaCl; brilliant cresyl blue, 1-50,000; neutral red, 1-50,000; or methylene blue, 1-50,000 (Becker, 1926*a*). For further suggestions see McClung (1929).

Dark-field illumination is very useful for counting the flagella of living flagellates and for the study of various cytoplasmic inclusions.

Control of temperature may be accomplished by either (1) making the study in a warm room, (2) placing the microscope in a warm box, or (3) using a warm stage. Several types of electric warm stages which are reliable and convenient are on the market.

Estimating the number of flagellates may be done roughly by diluting the entire contents of the section of the digestive tract to be dealt with in a measured quantity of salt solution and mixing thoroughly; then a measured quantity of this is placed on the slide under a twenty-two millimeter square cover glass. Ten fields are then counted and an average taken (Hegner, 1923*a*). Ratcliffe (1928) made a 1-10 suspension of cecal contents and placed this on a Leitz blood-counting slide. Counting the number of flagellates in four groups of large squares gave the number in 0.1 cubic centimeter of a 1-10 suspension. This figure times 100 gave the number per cubic millimeter of the cecal contents.

B. *Temporary killing* for counting flagella and recognizing other details may be accomplished by diluting a small amount of intestinal or cecal contents with one of the following: (1) Lugol's solution, diluted with water to a cherry red color, (2) saturated alcoholic solution of iodine diluted in the same manner; (3) either of the above with the addition of 0.5% eosin; (4) 1% to 2% osmic acid; (5) Janus green B, 1-2000; or (6) the flagellar stain of Noland (1928).[1]

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[1] Noland's flagellar stain:

80 cc. of saturated solution of phenol in water
20 cc. of 40% solution of formaldehyde in water
4 cc. of glycerine
20 mgms. of gentian violet

Moisten the dye thoroughly with one cubic centimeter of water before adding the other ingredients. Mix a drop of the reagent with a drop of the culture or other material to be examined.

3. *Making permanent slides*

For additional information about slide making the reader is referred to McClung's (1929) *Handbook of Microscopical Technique* (Hoeber). The following suggestions may be of value:

A. *Wet films.* Before making smears for fixation, cecal and intestinal contents may require a little dilution in a saline diluent. A thin, even smear is spread out on a slide or cover and then placed in the fixative before drying can occur.

a. Fixation. The writer has shown (1921) that different fixatives give different results when subsequently stained in the same manner. It is therefore desirable to use a number of fixatives if a full knowledge of the animals is desired. For routine work, Schaudinn's sublimate-alcohol-acetic employed at about 40° C. is a satisfactory fixative. Comparable results may be obtained with Worcester's fluid, or sublimate-acetic. Picric acid mixtures, especially Bouin's fluid and Hollande's fluid are very useful and the writer has found the latter especially satisfactory for intestinal flagellates. Osmic acid alone (one to two per cent), applied either as liquid or as fumes, or chrom-osmic or chrom-osmic-acetic mixtures are all useful. Weak Flemming's fluid was found to preserve the parabasal body of *Tritrichomonas muris* better than other fixatives, although one per cent chromic acid and Flemming's stronger fluid without acetic were also useful.

b. Staining. The most satisfactory stain for cytological details is probably iron alum-hematoxylin. The most selective differentiation is usually obtained when the preparations are allowed to mordant and stain for from two to twenty-four hours. However, shorter periods give workable results. For example, one may mordant ten minutes in a two to four per cent solution of iron alum at 30° to 40° C., rinse in water, then stain for ten minutes in 0.5% hæmatoxylin at the same temperature, then rinse in water, destain in the iron alum, wash thoroughly in water, dehydrate, clear and mount, and obtain usable slides. Delafield's hematoxylin gives good results with some flagellates. The Feulgen method (Cowdry, 1928) is supposed to stain only chromatin (thymonucleic acid) and should be used to supplement other technique. Counter-staining is sometimes desirable and may be carried out with eosin, orange G, light green, acid fuchsin or Bordeaux red. Other polychromatic effects can be secured by using Mallory's connective tissue stain or by the wet method of using Giemsa's stain.

B. *Dried films.* These do not usually give results that are satisfactory for cytological details but may be very useful for counting flagella and for quick identification. Dried smears may be fixed in alcohol, then stained with Giemsa's stain, or Wright's or Leishman's stains may be used, as for blood films.

IDENTIFICATION OF SPECIES

1. *General statement*

Identification of intestinal flagellates involves not only a knowledge of their morphology but also of their life histories and host-

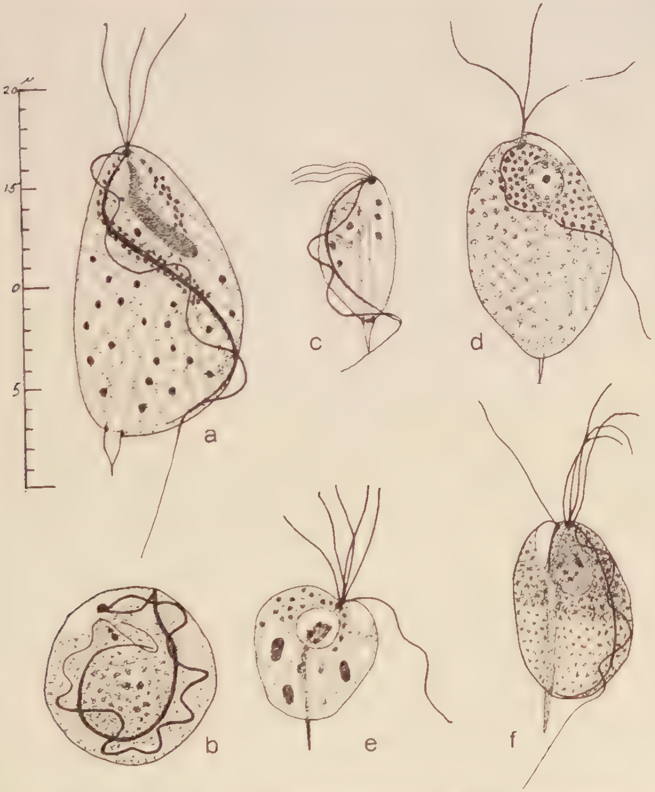

FIG. 12.—Flagellates of rats. a, *Tritrichomonas muris*, trophozoite; b, same, cyst-like stage; c, *Tritrichomonas minuta;* d, *Tritrichomonas parva;* e, *Hexamastix muris;* f, *Pentatrichomonas* sp(?).

parasite relationships. Species should, however, be recognizable morphologically before their status as distinct species is granted. The chief problems are (1) to determine the characters which differentiate any one species from others; (2) to determine the limits of variation for these characters; and (3) to determine the physiological characteristics as regards to host-parasite specificity and their relation to cultural and other conditions outside the host. In this section morphological features will be considered primarily. Host-parasite specificity is considered in another section.

2. *Key to genera and species*

The following key is based primarily on the characters that are revealed in adequately fixed and stained slides.

1(12) MONOZOA, without bilateral symmetry........................ 2

2(3) Body elongately pear-shaped, rounded anteriorly, tapering rather abruptly to a posterior spike or tail, no axostyle, cytostome large, containing an undulating flagellum, nucleus far forward, three anterior flagella, 10–18 μ long by 5–8 μ wide (Fig. 13, a) *Chilomastix bettencourti*

3(2) Body pear-shaped to spindle-shaped, with axostyle, TRICHOMONADIDÆ 4

4(11) Posterior flagellum attached to body by a spirally wound undulating membrane 5

5(10) With three anterior flagella, genus *Tritrichomonas*............ 6

6(9) Anterior flagella about half as long as body; undulating membrane and chromatic basal rod well developed; axostyle hyaline, of uniform diameter through the body, tapering rapidly to a sharp point at the posterior end, with chromatic ring just above the point of emergence from the body....................... 7

7(8) Body 8–20 μ long, inner and outer rows of chromatic granules well developed; late prophases show six double chromosomes besides the waning karyosome (Fig. 12, a)....*Tritrichomonas muris*

8(7) Body 3–10 μ long, inner and outer rows of chromatic granules not well developed, late prophases show 3 (or 4?) chromosomes (Fig. 12, c)..............................*Tritrichomonas minuta*

9(6) Body 5–11 μ long; three anterior flagella as long as or longer than the body; undulating membrane not well developed, usually sharply spiral in position not extending beyond the middle of the body; no chromatic basal rod; anterior third or half of body usually packed with chromatic granules, obscuring the nucleus; nucleus, when visible, has prominent, usually central round karyosome; axostyle slender, tapering gradually to the posterior end which may protrude for ¼ to ⅓ its length beyond the body; no chromatic ring at point of emergence (Fig. 12, d)..............................*Tritrichomonas parva*

10(5) Five anterior flagella, one group of four attached to one blepharoplast and a single one attached to a separate blepharoplast; undulating membrane extends the full length of the body, but not nearly so high as in *T. muris;* chromatic basal rod slender; axostyle slender, tapering gradually to the posterior end which may protrude ¼ to ⅓ its length beyond the body; no chromatic ring at the point of emergence; body 7–12 μ long (Fig. 12, f)
Pentatrichomonas sp., probably *P. ardin-delteili*

11(4) Posterior flagellum not attached to body by an undulating membrane; 5 anterior flagella besides the trailing flagellum; axostyle slender, tapering gradually to the posterior end which may protrude ¼ to ⅓ its length behind the body; no chromatic ring at point of emergence; 5–12 μ long (Fig. 12, e)
Hexamastix muris

12(1) DIPLOZOA, with bilateral symmetry............................ 13

13(16) With large anterior sucking disk, genus *Giardia*.............. 14

14(15) Body 7.4 to 13 (ave. 10.11) μ long by 5.4–9.8 (ave. 7.32) μ wide; ratio L/w 1.38 (Potter, 1928); parabasal bodies short, longitudinal and rounded at ends (Fig. 13, c)........*Giardia muris*

15(14) Body 9.5–18.8 (ave. 14) μ long by 5.13–10 (ave. 7.37) μ wide; ratio L/w 1.90 (Potter, 1928); parabasal bodies obliquely transverse, slender, comma-shaped (Fig. 13, d)
Giardia lamblia (*G. intestinalis, G. enterica, G. simoni*)

16(13) With no evident anterior sucking disk, body more slender, size smaller, genus *Hexamitus*................................. 17

17(18) Body 7–9 μ long by 2–3 μ wide, axostyles not especially chromatic, widely separated, no sharp caudal point (Fig. 13, e)
Hexamitus muris

18(17) Body 7–10 μ long by 3–5 μ wide, axostyles chromatic, apparently fused through most of their length; sharp caudal point (Fig. 13, f)...................................*Hexamitus pulcher*

3. *Some problems of flagellate organization*

There are many interesting problems in connection with the organization of these intestinal flagellates, only a few of which will be mentioned. For example, satisfactory conclusions have not been reached in regard to the real nature and functions of the axostyle, the chromatic basal rod, the parastyle, the parabasal body or the various groups of chromatic granules. Since Grassé (1926) has given an extended discussion of these problems, it will be sufficient to discuss them very briefly here.

The axostyle. In the trichomonads of the rat there are two types of axostyle. *T. muris* and *T. minuta* have axostyles that are hyaline, of uniform diameter through the body, tapering rapidly to a sharp point at the posterior end which normally projects very little beyond the body, and they exhibit a ring of chromatic material just anterior to the point of posterior emergence. In *T. parva, Pentatrichomonas* and *Hexamastix,* the axostyles tend to be more slender, tapering gradually through the body to the posterior end which is inclined to protrude considerably beyond the body. There is no posterior chromatic ring, but sometimes these axostyles stain rather deeply with hematoxylin. The significance of these differences is entirely unknown. Many other differences in axostylar organization occur in many species of trichomonads in other hosts, and need to be more thoroughly studied.

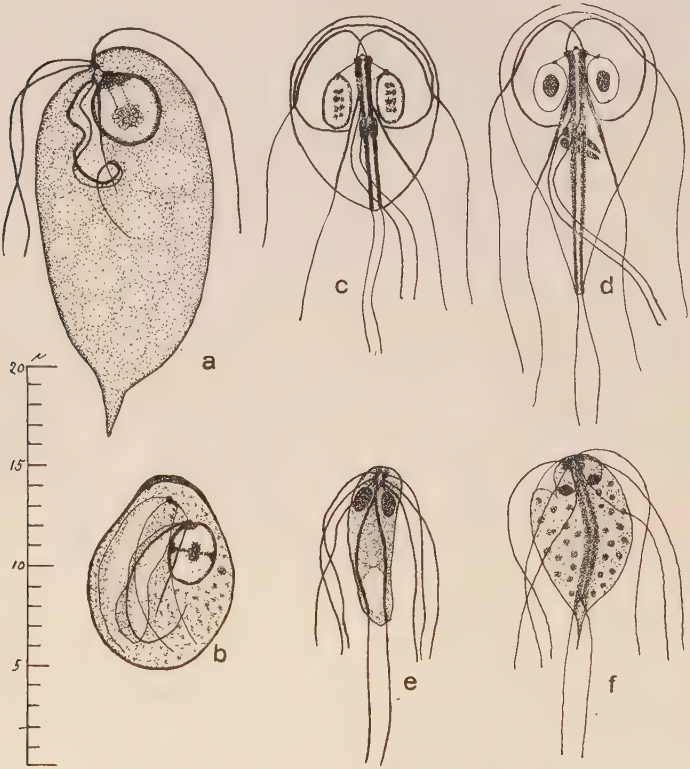

FIG. 13.—Flagellates of rats. a, *Chilomastix bettencourti, trophozoite;* b, same, cyst; c, *Giardia muris;* d, *Giardia lamblia*(?); e, *Hexamitus muris;* f, *Hexamitus pulcher.*

In *Chilomastix* there is no axostyle comparable to those of the trichomonads, although Kofoid and Swezy (1920) consider the parastyle to be homologous to one of the axostyles of *Giardia.* Grassé (1926) believes the parastyle to be an axostyle arrested in development, but does not regard the so-called axostyles of *Giardia* and *Hexamitus* as true axostyles, but as thickened and chromatic intracytoplasmic portions of the posterior flagella.

As to function, the axostyles have been regarded by many as primarily skeletal (*e.g.,* Wenyon, 1907), or as an organ of fixation (*e.g.,* Kuczynski, 1914), or as locomotor and the equivalent of an intracytoplasmic flagellum (*e.g.,* Kofoid and Swezy, 1915). Generalizations regarding the homologies and functions of all the structures that have been termed axostyles are probably unwarranted, each kind needing its own interpretation.

The chromatic basal rod is a chromophyllic rod at the base of the undulating membrane. It varies in length, thickness and shape in different species and may be replaced by a row of chromatic granules (*T. parva*). It is absent from the *Trichomastix* group of trichomonads. Grassé (1926) states that it is not composed of chromatin, although it stains heavily with hematoxylin. It is usually regarded as having some function relating to the activities of the undulating membrane, but its physiology has not been fully studied. In *Chilomastix* the parabasal of Kofoid and Swezy (1920) may be homologous to the chromatic basal rod of trichomonads, as may also the chromatic peristomal fibers of *Giardia.*

Chromatic granules in various groupings are common in trichomonad flagellates. More frequently there are one or more rows just beneath the chromatic basal rod and a row or group along the axis of the body, beginning at the blepharoplast. These are well developed in *T. muris,* less so in *T. minuta.* The more peripheral group appears to be absent in *Pentatrichomonas* and *Hexamastix,* but in the former there is a chromatic streak on the ventral side of the axostyle just back of the blepharoplast. In *T. parva* the chromatic granules are crowded into the anterior third of the body, usually with no definite arrangement except a row under the undulating membrane. The significance of these granules is quite obscure, but the axial group attached to the blepharoplast may constitute the material which aids in the generation of new axostyles during fission after the parental axostyle has degenerated. In some species (*Pentatrichomonas,* Fig. 12, f.) the anterior part of the body may stain more heavily than other portions. This cloud-like chromatic area may represent material comparable to the chromatic granules of *T. parva* which has not become organized into discrete granules.

The parabasal body. A great deal of confusion exists as to the nature and function of the structures to which this term has been applied. In the trichomonads the parabasal appears to be dissolved by certain fixatives (*e.g.,* Schaudinn's fluid) and to be preserved by others (osmic acid and certain of its mixtures). Duboscq and Grassé (1925) call attention to the heterogeneous nature of the parabasal and Grassé (1925) suggests that it may have a secretory function. Boeck (1919) believed that the parabasals of *Giardia* (which are not dissolved by Schaudinn's fluid) secrete glycogen, a reserve food supply utilized during the reproductive period and during encystment. Further study will doubtless show that the functions of the parabasal bodies are somewhat different in different flagellates.

CYTOLOGY AND LIFE HISTORY

The life history of none of the flagellates of rats has been completely worked out. The trophozoites, the dividing animals and the cysts are the only stages definitely known. Division is usually of the binary type but may be multiple. Both types of fission may occur in the active state or in the cyst.

1. Binary and multiple fission

Binary fission has been described for *Tritrichomonas muris* in considerable detail (Wenyon, 1907; Kuczynski, 1914, 1918; Kofoid and Swezy, 1915; and Wenrich, 1921). In the active state fission is accompanied by a mitotic division of the nucleus during which six double or split chromosomes form and divide while the karyosome gradually fades out. The behavior during division of the parabasal body, the chromatic granules and the cytostome has not been followed in detail.

Binary fission has not been worked out for any of the other trichomonads of the rat. However, the writer noted in 1921 that *T. minuta,* which resembles *T. muris* in most respects except in size, appears to have only three chromosomes in division. The idea that *T. minuta* may be a haploid race of *T. muris* has suggested itself and should be investigated.

Division of the nucleus by mitosis and the definiteness and constancy of the chromosomes in *T. muris* indicate that the mechanism for the qualitative division of the physical basis of heredity is as precise as it is in the higher animals and plants. Since the karyosome disappears during mitosis, to form again after nuclear division, a certain amount of nuclear reorganization must accompany each division. Perhaps this reorganization may serve to revitalize the nucleus making syngamy unnecessary.

Multiple fission has been described for *T. muris* by Kofoid and Swezy (1915) and consists of successive duplications of the nucleus and other organelles without immediate plasmotomy. This leads to the production of smaller individuals than those produced by binary fission, as many as eight individuals having been seen in one "somatella." This process seems to be of rare occurrence and has been seen only once by the writer. The significance of multiple fission is obscure. One is tempted to think of the formation of gametes (*T. minuta?*) in this manner but evidence of syngamy is entirely wanting, at present.

Adequate accounts of fission in *Chilomastix bettencourti* are lacking. Some details have been worked out for division of *C. mesnili* in the cysts (Kofoid and Swezy, 1920; Hegner, 1923*b*) and for the active stages of *C. aulostomi* (Belar, 1921), *C. magna* (Becker, 1926*c*) and of *C. gallinarum* (Boeck and Tanabe, 1926). The details of division within the cysts and that of the active state differ considerably and further studies should be made to determine all the facts and their adequate interpretation.

In *Hexamitus muris* binary and sometimes multiple fission takes place in the active state or within the cyst. Some stages are given by Wenyon (1907) but full details have not been described, for this species or for *H. pulcher. Alexeieff* (1911*b*) gives some stages in the division of *H. pulcher* from *Triton cristatus* but the most complete accounts in this genus are those of Swezy (1915) for *Hexamitus ovatus* and of Davis (1926) for *Octomitus* (*Hexamitus?*) *salmonis.* In each case intranuclear spindles with chromosomes are described.

Binary fission has been described in some detail for *Giardia muris* by Kofoid and Christiansen (1915*b*), for *G. microti* by Boeck (1917) and for *G. enterica* (*lamblia*) by Kofoid and Swezy (1922). In each case nuclear division within the parental nuclear membrane and the formation of four chromosomes and their division on a spindle were described. The axostyles appear to split while new flagella grow out to complete the normal number. Multiple fission was also described for both the active state and for cysts of *G. muris* by Kofoid and Christiansen, for the cyst of *G. microti* by Boeck, and the cyst of *G. enterica* by Kofoid and Swezy. In multinucleate cysts there were often two sizes of nuclei, suggesting maturation but no reduction of chromosome number was observed. The interpretation of these multinucleate cysts (up to sixteen nuclei) with only two or four axostyles remains obscure, especially since never more than two animals have been seen in a cyst.

2. *Encystment*

Encystment in trichomonad flagellates is a subject about which there is much difference of opinion although cysts regularly occur for *Chilomastix, Hexamitus* and *Giardia. Tritrichomonas muris* tends to round up soon after the death of the host unless kept at the body temperature of the host. The rounding-up process passes through a stage during which the anterior flagella and the undulating membrane remain active, causing the animals to rotate. Eventually, however, a stage is reached, the surface of which is smooth, and the organelles become entirely withdrawn within the cytoplasm. In fixed and stained preparations these completely rounded stages stain more intensely than trophozoites, indicating the more condensed condition of the protoplasm that characterizes many cysts. Furthermore the contained organelles appear to be entirely normal, degeneration therefore not being indicated. Occasionally similar fully developed spherical stages are found on slides prepared immediately after the death of the host, when the great majority of the individuals retained their normal vegetative shape, suggesting that such stages are a part of the life cycle and not always mere reactions to a changed environment. The writer has therefore been led to believe that these completely rounded stages are the biological equivalents of cysts although he has thus far been unable to demonstrate a true cyst membrane. A number of other investigators have interpreted these completely rounded up stages of *T. muris* as cysts, for example, Wenyon (1907), Kuczynski (1914) and Mayer (1920).

The *Chilomastix* of the rat forms a cyst (Fig. 13, b) that very closely resembles the cyst of *C. mesnili* of man. Division within these cysts has not been observed, but has been described for the cyst *C. mesnili* by Kofoid and Swezy (1920) and by Hegner (1923*b*).

Hexamitus muris forms cysts in the lower part of the small intestine and cecum. Binary and sometimes multiple fission appear to take place within the cysts soon after they are formed. Comparable cysts for *H. pulcher* have not been observed in the rat but probably occur.

Giardia encysts in the lower part of the small intestine and cecum of rats and the cysts may be found in the cecum, colon, and feces. Boeck (1919) found that there is a cycle of encystment of *Giardia* in rats with maximum numbers occurring at intervals of about seven days. A somewhat similar cycle was noted for the *Giardia* of man by Porter (1916). As already noted, both binary and multiple fission have been described for the cyst stage of *Giardia*.

The significance of encystment has been much in dispute. In these intestinal flagellates the cyst stage serves for the transfer of the animals from one host to another and also serves to permit reproduction by fission unhampered by the necessity of actively coping with the environment. It is an interesting biological speculation as to whether cyst formation has evolved primarily to serve the purposes of reproduction, reorganization or revitalization, or to serve primarily a protective function during transition through an environment unfavorable to normal vegetative activity. Since cysts for present day protozoa serve not only these two functions but may also serve as a retreat during the digestion of food, it is impossible to settle the problem as to whether any one of these functions had priority in evolution.

HOST-PARASITE RELATIONS

1. Relation to normal host environment

In order for any intestinal flagellate to establish itself in the digestive tract of a rat it must first gain entrance to that part which is its normal habitat and then be able to maintain itself in that situation.

In discussions of host-parasite relations the invasive powers of the parasite are often spoken of in contrast to the means of protection possessed by the host. As Hegner (1927*a*) has pointed out, intestinal protozoa gain entrance to a new host through the activities of the host itself. The protozoa are discharged from an infected host in the feces and if by chance infective stages contaminate the food or drink of another host, invasion is accomplished and they are taken into the new host's stomach. Here they are supposed to meet a barrier—a means of protection possessed by the host—in the gastric juice with its acidity and protein digestive powers. No flagellate is known to colonize itself in the stomach of a rat, but they do establish themselves in the small intestine and cecum, hence must pass through the stomach.

The invading flagellates may succeed in passing the stomach barrier either by the aid of special protective membranes (cysts) or by the aid of natural powers of resistance without special membranes. *Chilomastix, Hexamitus* and *Giardia* are known to produce cysts, but similar cysts are not well established for the trichomonads. Hegner (1924) first showed that *T. muris* can pass through the stomach of a rat and into the small intestine in a viable state without encystment. This conclusion was confirmed by Wenrich and Yanoff (1927) and extended to include *T. parva* and *T. minuta*. The latter authors also showed that *Pentatrichomonas* of man, which is probably the same as that in the rat, could pass the stomach barrier of the rat.

The above considerations suggest the following problems: (a) for the flagellates in the vegetative state: (1) what conditions are necessary for viability outside the host (Hegner, 1928*a*, has made such a study for *Trichomonas* of man)? (2) is the scarcity of cysts in the trichomonads to be attributed to an acquired resistance to the gastric juice of the host? (3) how does this resistance compare with that of flagellates which regularly form cysts? (4) what range of hydrogen-ion concentration can trophozoites withstand? (5) what range of strengths of gastric enzymes can trophozoites withstand? (6) what variations in these two conditions (acidity and enzyme concentration) occur in the host, *i.e.*, can the flagellates invade any host any time, or does invasion require special conditions in the host? (7) what is the effect of sudden changes of hydrogen-ion contraction on the flagellates (entrance to stomach and passage from stomach to small intestine)? (8) how are trophozoites affected by the secretions they meet in the small intestine (bile, pancreatic juice, secretions from the small intestine)? (9) what are the physiological differences among the different species causing some to colonize themselves in the small intestine and others in the cecum?

(b) For flagellates in the encysted state: (1) what are the chemical and physical properties of the cyst membrane for each species? (2) what conditions are necessary for survival of the cyst stage outside the host? (3) how long may the cyst survive outside the host under favorable circumstances? (4) what effect, if any, does gastric juice and its constituents have on the cyst stage? (5) where and how does excystation normally occur? (6) what conditions in the host's digestive tract are favorable or unfavorable to excystment and the survival of excysted animals? Hegner (1927*b*) believes that moisture and a temperature of 37° C. for several hours are the only factors necessary to bring about excystation of intestinal protozoa of man. Perhaps the same is true of those in the rat. According to Hegner (1927*d*) cysts of *Giardia lamblia*, when injected into the stomach of a rat were found (1) to show motility sooner when placed at 40° C. than control cysts, (2) the percentage of viable cysts was greater in the controls, indicating that some had been killed, and (3) that movement and excystation were to some extent inhibited. Cysts remained viable for as long as six hours in the rats' stomachs.

In regard to the defenses of the host several problems suggest themselves: (1) what habits of the host tend to increase or minimize the likelihood of contamination of food or drink with viable stages of intestinal flagellates from feces? (2) what variations in the character of the host's secretions occur which might affect the survival of ingested flagellates? (3) does the host have any natural resistance to invasion other than that implied by the effect of secretions? (4) does the host develop an acquired immunity, either partial or complete, as a reaction to the invasion of any of the flagellates? (Lavier, 1921, states that Deschiens infected mice with *Giardia* from man and that the mice developed an immunity to this species of *Giardia*, and Davis (1926) believes that young trout develop an immunity to *Octomitus salmonis*.) (5) Having successfully invaded the normal habitat in the host, what conditions of the host's digestive tract affect its survival? (The relation to the diet of the host is treated in Chapter XI.)

The food habits of the intestinal flagellates of rats have not been thoroughly investigated. *T. muris* and *T. minuta* do not usually contain solid food bodies but contain vacuoles filled with fluid substances, the exact nature of which is unknown, but a fluid diet is suggested. *T. parva, Pentatrichomonas* and *Hexamastix* frequently have food vacuoles containing bacteria, indicating the nature of their diet. The *Pentatrichomonas* of man is known to ingest red blood corpuscles (Haughwout and De Leon, 1919; Kofoid and Swezy, 1923), but this has not been observed for the one in the rat. *Chilomastix* ingests bacteria of several kinds but little is known as to possible limitations on the kind that it needs or as to its ingestion of other than solid food. *Giardia* apparently lacks a cytostome and so does *Hexamitus muris*. They are supposed to nourish themselves osmotically. *Hexamitus pulcher* frequently shows a slit-like opening at the anterior end in prepared slides but ingestion of food has not been demonstrated. The cytoplasm is rather regularly filled with refractive granules which Becker (1926*c*) took to be mitochondrial in nature, but which usually stain with hematoxylin after such fixatives as Schaudinn's fluid. Their exact nature is not known but they may be concerned with metabolism.

Parasites, especially the vegetable organism, *Sphærita,* attack the flagellates of rats, often in considerable numbers. They have been seen in *Chilomastix* and the trichomonads but not in *Giardia* or *Hexamitus*. There is much variation in the size and appearance of these parasites, suggesting that there may be several species of them. Becker (1926*b*) has described some stages in the life history of a *Sphærita* in *Endamœba citelli,* but further studies are desirable.

These flagellates are also the prey of other protozoa of the digestive tract, especially of the endozoic amœbæ, as figured by Wenyon (1907) and Kuczynski (1914) and as observed by the writer.

2. Pathogenicity

Little is known as to the possible pathogenic effect of intestinal flagellates on rats. So far as the writer is aware there is no evidence for the pathogenicity of any of the trichomonads except *Pentatrichomonas*. In many cases where this species is present in any considerable numbers the contents of the cecum contain many leucocytes, and it is of importance to determine whether this condition is due to the presence of *Pentatrichomonas* as seems to be indicated. Kessel (1928*b*) inoculated kittens with *T. muris* and "*T. parva*." Only "*T. parva*" produced an infection in the kittens but definite symptoms of diarrhea and even fatal dysentery were produced. Pathological conditions resulted whether the infection was a natural one or an experimental infection from other kittens, from man, from the monkey or from the rat. The writer believes that Kessel's "*T. parva*" is the *Pentatrichomonas* of the rat since his figure resembles that species in all respects except in the number of flagella and they are usually difficult to count. The writer also believes that his trichomonads of man, monkey and kittens are this same *Pentatrichomonas*. Wenyon (1915) says, "Dr. A. C. Stevenson has shown me a section of the cæcum of a mouse in which there is a definite lesion of the mucous surface which is being invaded by numerous *Trichomonas*." (The species is not mentioned but may be *Pentatrichomonas*.)

Phagocytosis of *Trichomonas felis* by polynuclear leucocytes has been reported by Cunha and Muniz (1922) and they interpret this phenomenon as a protective action on the part of the host. The writer has seen the same phenomena in the living condition, that is, a *Pentatrichomonas* of the rat ingested by a leucocyte, and in prepared slides has seen the *Pentatrichomonas* of man that had been ingested by leucocytes. Perhaps the *Trichomonas felis* of Cunha and Muniz is the same as the *Pentatrichomonas* of man and the rat.

The *Chilomastix* of the rat is, apparently, without any pathogenic effect upon its host, but no extensive studies have been made to determine this point.

Giardia was believed not to cause diarrhea in rats by Boeck (1919). The same author denied any pathological effects on meadow mice by *G. microti* although Kofoid and Christiansen (1915*a*) believed that *G. microti* caused inflation and discoloration of the intestine of mice and that *G. muris* (1915*b*) caused discoloration and flaccidity in the intestine of mice at the point of infection. *Giardia* is definitely pathogenic to rats and mice according to Fantham and Porter (1916) and Galli-Valerio (1924). The possible pathogenic rôle of the different species of *Giardia* in the rat needs further investigation.

3. *Relation to other hosts*

One of the most interesting problems in parasitology is that pertaining to host-parasite specificity. Hegner (1928*c*) believes that each species of parasite is, as a rule, limited to one species of host, while Kessel (1928*a*) believes that transfer of intestinal protozoa from one species of host to another is more common than is generally supposed. As to the intestinal flagellates of rats, not a great deal has been determined by experiment, so that an inviting field of inquiry is open.

Since the common rats, especially the Norway rat, have become widely distributed over the earth, they have doubtless carried their flagellates with them, so that these flagellates have an equally wide distribution, but there are many unanswered questions as to the transferability of these flagellates to other animals, including man.

Tritrichomonas muris (Galli-Valerio, 1907) has been found by the writer in albino and wild Norway rats, in the wild black rat, in albino and wild gray house mice and in the field mouse *(Peromyscus leucopus)*. It has been reported from rats and mice in many parts of the world. What appears to be the same species is reported by Becker (1926*c*) from the ground squirrel (*Citellus tridecemlineatus)*. More studies are needed to determine its host distribution. At present it appears to be limited to rats and mice and the ground squirrel.

Tritrichomonas minuta Wenrich (1924) has been found by the writer in albino and wild Norway rats, in albino and wild gray house mice and in the field mouse *(Peromyscus leucopus)*. It appears to be more common in mice than in rats. Since this species is not generally recognized, its host distribution is unknown except for the statement above.

Tritrichomonas parva Alexeieff (1911*a*), has been found by the writer only in albino and wild Norway rats and it was from a rat that Alexeieff first described it as a new species. Kessel's "*T. parva*" is probably *Pentatrichomonas*. Further studies are necessary to determine its host distribution.

Pentatrichomonas has been found by the writer only in albino and wild Norway rats, but is believed to be identical with the species found in man. Kessel (1928*b*) transferred what is believed to be this species *("T. parva")* from the rat to kittens where it produced definite pathological symptoms. Kofoid and Swezy (1924) state that the *Trichomonas* of man "may more readily be detected by feeding experiments with flagellate-free rodents than by stool examination in light infections." It is probable that the *Trichomonas* transferred by Hegner (1928*c*) from the cecum of the rat to chicks was *Pentatrichomonas*. Chatterjee, Harendranath and Mitra (1927) have recently described a *Pentatrichomonas* (*P. canis-auri*) from an Indian jackal which may be the same species. The available evidence indicates that this *Pentatrichomonas* has a wide distribution among mammals and that it is pathogenic to many of them.

Hexamastix muris Wenrich (1924) has been found by the writer thus far only in the wild black rat (*Mus rattus*). Further studies will doubtless reveal a wider distribution. Its morphology is very similar to that of the *Tetratrichomastix citelli* of the ground squirrel described by Becker (1926*c*).

Chilomastix bettencourti Fonseca (1915), or what is supposed to be this species, has been found by the writer in albino and wild Norway rats, in the wild black rat, in the house mouse and in the field mouse (*Peromyscus leucopus*). A similar species has been reported for various wild rodents, as, for example, in *Gerbillus pygargus* by Chalmers and Pekkola (1918), and in *Lateroma* (*Tatera*) *lobengula* and *Mus coucha* by Fantham (1925) in Africa. Wenyon (1926) states that he sees no difference between the *Chilomastix* of man and that of rodents and Kessel (1928*a*) says that he has successfully transferred the *Chilomastix* of man to monkeys and the domestic pig. It will require a great deal of carefully controlled transfers and a critical study of characters to determine the extent to which the *Chilomastix* of the rat can colonize itself in other hosts, including man.

Hexamitus muris (Grassi, 1882) has been recorded from many different kinds of rats and mice and is apparently widely distributed through this group of rodents. Its possible occurrence outside this group needs to be investigated. Wenyon (1926) suggests that it may be the same as the fresh water species since it appears in water to which feces containing its cysts have been added. Further studies of this possibility are much to be desired.

Hexamitus pulcher Becker (1926*c*) was first described from the rat by Prowazek (1904) as *Octomitus intestinalis*. The writer has found it in both the albino and wild Norway rat and in both the house mouse and field mouse. It appears to be more common in mice than in rats. Becker (1926*c*) has described this species from the ground squirrel in Iowa and the writer has found it in a ground squirrel at Flagstaff, Arizona. What appears to be the same species was noted by Alexeieff (1911b) in a salamander and the writer has observed the same organism in various AMPHIBIA, both ANURA and URODELA. Studies of the host-parasite distribution of this species should be especially interesting since it seems to have established itself in both warm-blooded and cold-blooded hosts.

Giardia muris (Grassi, 1879) Bensen (1908) has been reported for a number of species of rats and mice but a wide distribution among other hosts has not been indicated. A second species of *Giardia* has been noted by Boeck (1919), Simon (1922), Lavier (1924), Nieschulz and Krijgsman (1925) and Hegner (1927*c*) and has recently been shown by Potter (1928) to be morphologically identical with the *Giardia* of man. Hegner (1927*c*) has demonstrated that the *Giardia* of man can excyst and colonize itself in rats. The rat therefore does harbor the *Giardia* of man, but to what extent is not known. It is desirable to determine if other hosts may harbor the species found in man, or if man may not sometimes harbor species other than *G. lamblia*.

CHAPTER XV

HOST-PARASITE SPECIFICITY IN THE GENUS *GIARDIA*

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INTRODUCTION

THE genus *Giardia* is of particular value for the study of host-parasite specificity since each species of host seems to be infected by a distinct species of parasite. The *Giardia* of man was first described by Leeuwenhoek in 1681 (Dobell, 1920). Giardias were reported from mice, rats and cats by Grassi (1879-1881). Metzner (1901) gave a good description of giardias from the rabbit. All of these giardias were supposed to belong to a single species and cross-infection between different species of hosts in which they were found was supposed to be the rule. Bensen, however, in 1908 distinguished three species which we now know as *Giardia lamblia* from man, *Giardia muris* from rats and house mice, and *Giardia duodenalis* from the rabbit. Practically all protozoölogists who accepted these as separate species still believed until quite recently that cross-infection occurred and it is even supposed by many physicians at the present time that human infections are due to contamination of foód or drink with cysts from rats or mice.

Twenty or more species of *Giardia* have been described, especially during the past fifteen years, on the basis of morphology or occurrence in a host species in which they had not previously been reported. The following table presents a list of these species with the name of the describer and date and that of the host. The validity of all these species is not certain. For example, *G. simoni* is probably *G. lamblia* that has succeeded in setting up a temporary infection in rats. *Giardia xenopi* may belong to the species *Giardia* *agilis* and the *Giardia* noted by Thomson (1925) in the nematode worm *Viannella* from the viscacha is probably the same species, named *Giardia viscaciæ,* which Lavier (1923) reported from the viscacha. It is the object of this chapter to discuss methods of obtaining and preparing material for study and problems and methods of procedure for the solution of the many questions involved in the host-parasite specificity of the members of this genus.

Species Name	*Name of Describer*	*Date*	*Host*
Mammals			
Giardia lamblia	Lambl	1859	Man
Giardia pitymisi	Splendore	1920	Vole
Giardia duodenalis	Devaine	1875	Rabbit
Giardia muris	Grassi	1879	Rats and mice
Giardia microti	Kofoid and Christiansen	1915	Field mice
Giardia canis	Hegner	1922	Dog
Giardia caviæ	Hegner	1923	Guinea-pig
Giardia viscaciæ	Lavier	1923	Viscacha
Giardia simoni	Lavier	1924	Rats
Giardia capræ	Nieschulz	1923	Goat
Giardia equi	Fantham	1921	Horse
Giardia bovis	Fantham	1921	Cattle
Giardia sp.	Hegner	1924	Wild cat
Giardia sp.	Deschiens	1925	Lion
Giardia cati	Deschiens	1925	Cat
Giardia sp.	Hegner	1924	Monkey
Giardia sp.	Nieschulz	1923	Calf
Giardia suricatæ	Fantham	1923	Meercat
Giardia beckeri	Hegner	1926	Ground squirrel
Giardia otomysis	Fantham	1926	*Otomys irroratus*
Giardia bradypi	Hegner and Schumaker	1928	Three-toed sloth
Birds			
Giardia sanguinis	Gonder	1910	*Elanus cæruleus* (blood)
Giardia ardeæ	Nöller	1920	Herons
Giardia sp.	Kotlán	1922	*Lanius collurio*
Giardia sp.	Kotlán	1922	*Recurvirostra avocetta*
Giardia sp.	Hegner	1925	Black-crowned night heron
Giardia sp.	Hegner	1925	Great blue heron
Reptiles			
Giardia varani	Lavier	1923	*Varanus niloticus*
Amphibia			
Giardia agilis	Kunstler	1882	Tadpole
Giardia xenopi	Fantham	1923	Clawed frog
Fish			
Giardia denticis	Fantham	1919	Fish (blood)
Nematode			
Giardia sp.	Thomson	1925	*Viannella* sp.

SOURCES OF SUPPLY

Surveys of the intestinal protozoa of man indicate that *Giardia lamblia* occurs in about ten per cent of the general population. As a rule cysts only are to be found in formed stools. They are very irregular in their appearance, being present in considerable numbers on one day but absent or few in numbers on other days. Trophozoites are passed when stools are loose and likewise are somewhat irregular in their appearance. Perhaps the easiest species to obtain is *Giardia muris* that occurs in rats and mice. Cysts may be found in the fecal pellets of these animals and uninfected rats may be infected by placing them in the same cage with an infected specimen. Heavy infections may be secured by adding infected pellets to the food supply of the rats, being careful to supply sufficient moisture to prevent the drying out of the cysts. Field mice are frequently found to be infected but are more difficult to obtain for study.

The trophozoites living in rats or mice may be secured without killing the hosts by giving them a dose of Epsom salts. Active flagellates can then be secured in the liquid fecal material that results from this treatment.

Giardias do not seem to be present in as many of the other common laboratory animals, such as rabbits, guinea-pigs, cats and dogs. The incidence of infection in domesticated animals such as horses, cattle, sheep and goats is not well known. Thus far, giardias have been reported from a number of species of birds but only two species have been given names, one from the blood of a falcon, *G. sanguinis* (Gonder, 1910) and *G. ardeæ* from the intestine of several species of herons (Nöller, 1920). Giardias have also been recorded from the intestine of a shrike, an avocet (Kotlán, 1922) and from the intestine of the great blue heron and black-crowned night heron (Hegner, 1925). It is interesting to note that all of these birds feed upon animals that are known to be infected with giardias. Falcons and shrikes capture and devour field mice; avocets and herons feed on tadpoles. Perhaps those species found in birds have evolved from those that occur in their prey. Of the giardias that have been reported from cold-blooded animals, only *G. agilis* of the tadpole appears to be common.

OBTAINING MATERIAL

The easiest method of obtaining specimens of *Giardia lamblia* for study is to secure fecal samples from a number of persons, especially those suffering from diarrhea. Thin smears made up with 0.7% NaCl should reveal cysts or trophozoites if present. Cysts in the living condition are very easy to identify. They are oval in outline, average about ten microns in length and seven microns in breadth and are characterized by the presence of several curved lines which may be seen extending through the protoplasm in a more or less longitudinal direction. The trophozoite can hardly be confused with any other type of organism that occurs in human feces. As shown in Fig. 14, they are broad, have a large sucking disc on the ventral surface and a pointed tail. They roll over and over in the medium without making much progress.

Cysts of the giardias of rats and mice, as already mentioned, may be obtained from the fecal pellets of these animals and trophozoites in the more liquid feces as the result of treatment with Epsom salts. The normal habitat of *Giardia* is the duodenum. Here in freshly killed animals trophozoites will be found in material squeezed out of a section of the duodenum on a slide and diluted with 0.7% saline solution, or in material from scrapings of the inner surface. If small pieces of the duodenum of a heavily infected animal are fixed in Bouin's or Zenker's solutions and cross sections made, giardias will be found in the crypts clinging to the epithelial cells with their sucking discs. The giardias of the other animals mentioned may likewise be secured either in the feces or in material taken from the duodenum of the freshly killed host. Specimens in various stages of encystation may be found in the jejunum, ileum, cecum and colon.

PREPARATION FOR STUDY

Cysts and trophozoites of the giardias available may be fixed in smears with Schaudinn's solution and stained with iron-hematoxylin. When prepared in this way they exhibit the various structures shown in Fig. 14.

Unfortunately giardias are among the few human protozoa that have never been cultivated in artificial media. Many attempts to do so have been made by a number of investigators in the writer's laboratory but thus far without success. It is very important that a method for the cultivation of members of this genus be worked out inasmuch as past experience has shown that the ability to cultivate an organism aids tremendously in the growth of our knowledge of that organism.

PROBLEMS FOR STUDY

a. Morphology of the trophozoite. Inasmuch as our conclusions with respect to host-parasite specificity in this genus depend upon our ability to recognize different species and since the recognition of species requires a detailed knowledge of morphology it is important for us to be familiar with the structure of both the trophozoite and the cyst. As indicated in Fig. 14, the trophozoite possesses a large ventrally located sucking disc. There are two nuclei present. These are attached by rhizoplasts to blepharoplasts at the anterior ends of the two axostyles. The writer believes that there are two thread-like axostyles, as indicated in the illustration, with a single blepharoplast at the anterior end of each. Certain investigators (Kofoid and Swezy, 1922) believe that there is a single axostyle and others (Wenyon, 1926) believe that there are two blepharoplasts at the anterior end of each axostyle. Further study is necessary before one or the other of these views is established. Between the blepharoplasts at the anterior end of the axostyles is an interblepharoplastic rhizoplast in the center of which is an interblepharoplastic granule. The four pairs of flagella are located as indicated in Fig. 14. The antero-lateral flagella extend forward from the blepharoplast and appear to cross near the anterior end. Kofoid *(1922)* suggests that a chiasma does not really exist at this point but that the two flagella simply come into contact without crossing. The shield-shaped areas on either side of the body are known as lateral shields. The region extending from the point where the posterior lateral flagella emerge from the body to the posterior end is often referred to as the tail. Near the center of the body are one or two deeply staining structures known as parabasal bodies.

b. Morphology of the cyst. Cysts of *Giardia* that are properly stained reveal two, four, eight or more nuclei which are distributed with two near the anterior end or two near either end or four near the anterior end or four near either end. Multinucleate cysts of both trophozoites and cysts of *Giardia muris* and *Giardia microti* have been described by Kofoid and Christiansen (1915). Within the cyst are a number of deeply staining fibrils. It is possible to identify some of these as axostyles and intracytoplasmic portions of the flagella that have persisted from the trophozoite stage, but other fibrils have not been identified with certainty. A careful study of encystation will be necessary in order to determine the origin of all of these fibrils.

c. Division of the trophozoite. The trophozoite of *Giardia* apparently divides by longitudinal binary fission. Stages in this process have been described by Wenyon and O'Connor (1917) and by Kofoid and Swezy (1922). There are still, however, many details that need elucidation. We owe descriptions of nuclear division particularly to Boeck (1917), who described mitosis in *Giardia microti* including the formation of eight chromosomes, and in *Giardia lamblia* to Kofoid and Swezy (1922). This work needs confirmation and more detailed studies of nuclear division in other species are desirable.

d. Excystation. Very little is known about excystation in *Giardia.* Fragmentary accounts of this process in the case of *G. lamblia* in the feces of a human host have been supplied by Simon (1921) and the writer (Hegner, 1925). It is possible, however, to feed cysts of giardias to laboratory animals such as rats and then kill the animals at intervals. The writer (Hegner, 1927) found that under these conditions *G. lamblia* did not excyst in the stomach or duodenum but mostly in the small intestine from thirty to sixty centimeters posterior to the stomach. The minimum time required for excystation was about thirty minutes. The excysted specimens appeared to remain in the portion of the intestine where excystation occurred, whereas unexcysted cysts were carried down the cecum.

Giardias are stimulated to activity within the cysts by raising the temperature; if they are placed in water or saline solution under a cover glass and kept in a warm chamber at about 40° C. for approximately an hour movements begin within the cyst wall but do not result in excystation. This method of stimulating activity within the cyst has one distinct advantage, however, in that it can be used to determine whether or not cysts are alive.

e. Cultivation. As noted above no one has succeeded in cultivating any species of *Giardia* outside of the body. Attempts have been made in the writer's laboratory by a number of investigators but without success. It should be remembered that giardias have been reported from several cold-blooded animals, including tadpoles of frogs. The incidence of infection in tadpoles seems to be high and hence this material is easy to obtain. Giardias of cold-blooded animals may be more easily cultivated than those of warm-blooded animals and hence experiments with *Giardia agilis* are indicated.

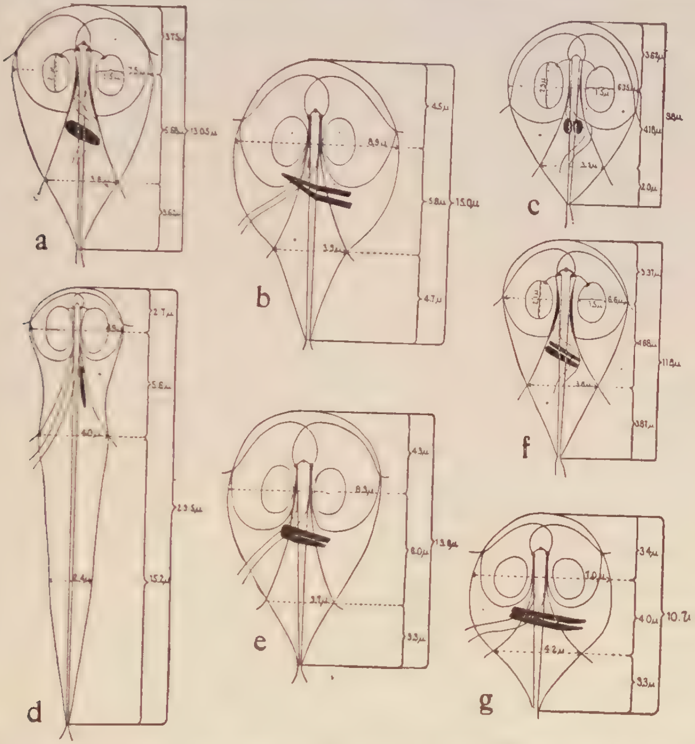
375μ
7.5μ
5.68μ
13.05μ
3.8μ
3.62μ
a
4.5μ
8.9μ
5.8μ
15.0μ
3.9μ
4.7μ
b
3.62μ
6.35μ
1.5μ
2.3μ
9.8μ
4.18μ
3.1μ
2.0μ
c
2.7μ
4.5μ
5.6μ
4.0μ
23.5μ
2.4μ
15.2μ
d
4.3μ
8.3μ
8.0μ
13.8μ
3.7μ
3.3μ
e
3.37μ
6.6μ
1.5μ
4.68μ
11.8μ
3.8μ
3.87μ
f
3.4μ
7.0μ
4.0μ
10.7μ
4.2μ
3.3μ
g

FIG. 14.—Giardias from man and lower animals. Diagrams showing the specific differences between giardias living in different species of animals. a, *G. lamblia* from man. b, *G. duodenalis* from the rabbit. c, *G. muris* from rats and mice. d, *G. agilis* from frog tadpoles. e, *G. canis* from the dog. f, *G. microti* from meadow mice. g, *G. caviæ* from the guinea-pig. × 2800. (a, c, f, after Simon; b, d, e, g, after Hegner.)

Until it is possible to cultivate giardias many problems will have to remain unsolved. Reference may be made here to the fact that the first definite success in the cultivation of an *Endamœba* was that of Barret and Smith (1923) who succeeded in cultivating *E. barreti* of a cold-blooded animal, the turtle, and to the great progress that has been made in our knowledge of *Endamœba his-*

tolytica since this species was successfully cultivated by Boeck and Drbohlav (1925).

f. Specific differences based on morphology. The genus *Giardia* is of peculiar interest because in almost every case each host species contains a distinct species of *Giardia* that can be distinguished morphologically from other species. In Fig. 14 are presented diagrams of seven species that show measurements and morphological characteristics of importance in diagnosis. The criteria used in separating these species are first those relating to size. Investigators who have made studies of size in protozoa know that size is a very constant characteristic. The measurements of importance are total length, not including the caudal flagella, breadth across the ventral sucker, ratio of length to breadth, the distance from the anterior end to the center of the nucleus, from the center of the nucleus to the end of the lateral shields, the distance from the end of the lateral shields to the posterior end of the body, and the distance across the body at the ends of the lateral shields. The contour of the body is of importance. In *Giardia lamblia* from man (Fig. 14, a), for example, the lateral shields taper gradually to their posterior ends, whereas in *Giardia duodenalis,* from the rabbit (b), and *Giardia caviæ,* from the guinea-pig (g), the lateral shields bulge outward. The distance of the nuclei from the median line and from the posterior edge of the sucking disc and their position in the body, whether parallel to the axostyle or obliquely situated, are of importance. The size and shape of the nuclei are also diagnostic characteristics of value. In giardias fixed in Schaudinn's solution and stained with iron hematoxylin the parabasal bodies are usually conspicuous. These bodies are very constant in number, shape and position. For example, *Giardia agilis* from the frog tadpoles has a single club-shaped parabasal body lying parallel to the axostyle. The *Giardia* from the rabbit (Fig. 14, b) has two long, slender parabasal bodies slightly bent near the center and lying obliquely across the axostyles and *G. muris* from rats and mice (Fig. 14, c) possesses two oval parabasal bodies that lie side by side and parallel to the axostyle. Another characteristic that may be of diagnostic value is the affinity for iron hematoxylin stain. The writer (Hegner, 1925), for example, found it very difficult to stain the giardias from birds by methods that produced beautiful preparations of specimens from other hosts. By means of the criteria set forth it seems possible to distinguish various species of *Giardia*. A mere glance is sufficient to distinguish such species as *G. agilis* from the tadpole and *G. muris* from rats and mice. A more careful study is necessary for the recognition of other species. No doubt giardias occur in many species of animals in which they have not yet been found. They should be looked for in the duodenum. Rodents seem to be infected most frequently and careful studies of the giardias in nearly related species of rats, mice and other rodents are indicated. The situation as regards the giardias of birds is in a very chaotic condition and examination of birds of prey, shrikes, water birds and other types that feed largely on small animals would no doubt yield valuable results.

g. Cross-infection experiments. This subject is treated in general in another chapter (Chapter IX). Certain investigators are not willing to accept the type of statistical work just described as sufficient for separating species of *Giardia*. The belief has been expressed that the differences observed may be due to differences in environment in different species of hosts and that one species of *Giardia* may, therefore, live in several species of hosts and exhibit slightly different but constant characteristics in each host. In order to settle this point cross-infection experiments are necessary. The writer admits that such experiments are desirable and hopes that they will be carried out by competent investigators in the near future. The difficulties involved, however, are considerable and only under exceptional conditions can one obtain parasite-free animals and a sufficient supply of giardias for such work.

Of particular interest is the situation regarding the possible infection of the rat with *G. lamblia* from man. Boeck (1919) mentions the discovery in a laboratory rat of a type of *Giardia* resembling *G. lamblia*. Simon (1922) encountered similar specimens in two laboratory rats which he considered to be of the same type as those reported by Boeck. Lavier (1924) reports three out of 301 wild rats collected in the sewers of Paris with giardias morphologically like *Giardia lamblia*. Since he could not infect laboratory rats with human giardias, Lavier decided that he was dealing with a distinct species which he named *G. simoni*. Giardias resembling *G. lamblia* were also described from rats by Nieschulz and Krijgsman (1925). Potter (1928) found two types of giardias living together in the same laboratory rats. He concludes from a morphological study involving specimens of *G. muris, G. microti* and *G. lamblia* that he was dealing with *G. muris* and *G. lamblia* and that his rats were actually infected with giardias from man. It is not certain that Potter was dealing with what may be called a permanent infection. The writer (Hegner, 1927) encountered *lamblia*-like giardias in two laboratory rats that were being used for studies of excystation. Later, attempts were made to infect rats by feeding them cysts of *G. lamblia*. Temporary infections were apparently established in four rats; these infections were highest on the sixth and seventh days, decreased by the twelfth and fourteenth days and disappeared by the sixteenth and nineteenth days. The trophozoites recovered from these animals were smaller than those of *G. lamblia* from man; this may have been due to their unusual habitat. No cysts were passed by the experimentally infected rats which suggests that the rat is not an important transmitting agent of the human *Giardia*. Extensive and important experimental work of a similar nature could be carried out with giardias from man and a number of lower animals.

h. Transmission. It seems probable that the cyst is the infective stage of *Giardia*. The trophozoites might be able to reach the duodenum in a viable condition (Hegner, 1926) but the chances that they would live outside of the body long enough under ordinary conditions to be ingested by another host are very slight. Experiments have been carried out to determine resistance of cysts of *Giardia* to various factors such as temperature (Boeck, 1921) encountered outside of the body of the host. The work of Stiles and Keister (1913), Wenyon and O'Connor (1917), and Root (1921) indicates that flies may be important transmitting agents; and several investigators have implicated the cockroach (Porter, 1919; Pessva, 1927). Further experiments regarding the viability of both trophozoites and cysts of giardias outside of the host and of their transmission by insects are desirable.

i. Age resistance. A number of investigators, especially Dobell (1921), Maxcy (1921), and Simon (1924), have published data which indicate that the resistance to infection with *Giardia lamblia* is much higher in children than in adults. Whether the young of other animals are more frequently infected than the adults is not known. A study should be made of this problem. It is customary to speak of age resistance when facts of this type are revealed but the factors involved in age resistance are practically unknown. Perhaps, as Dobell suggests, the higher incidence of giardias among children may be due to the greater ease of finding the organisms in their stools. Perhaps changes in diet are responsible (Hegner, 1923). The problem is open to experimental study.

j. Pathogenicity. The terms lambliasis, giardiasis, and flagellate diarrhea have been applied to pathological conditions supposed to be due to infections with giardias. It has never been satisfactorily established that the giardias are actually responsible for the conditions observed. Many physicians who have been unable to find any other etiological agent present believe that *Giardia* is responsible. Giardias do not penetrate the intestinal wall. They do, however, cling to the epithelial cells with their sucking discs. Jassinowsky (1927) estimated that over 37,000,000 giardias were present in the jejunum of a single rabbit, that is, over 1,000,000 per square centimeter of mucous membrane. Such large numbers might bring about a diarrheic condition as the result of irritation or, as Haughwout (1918) has suggested, of interference with the functions of the glands.

In recent years a large number of reports have been published implicating giardias in the production of cholecystitis. Case reports on this subject do not enable one to come to a definite conclusion. The reader is referred to current numbers of the *Tropical Diseases Bulletin* for abstracts of work being published on the subject.

A fact that may have a bearing on the pathogenicity of *Giardia* is the discovery of red blood cells inside of the body of giardias by Knowles (1928). The presence of red blood cells in a parasitic protozoön is usually accepted as evidence of pathogenicity. That this is not true in the case of trichomonad flagellates has been shown by the writer (Hegner, 1928*c*). How the red blood cells were ingested by giardias is difficult to understand.

CHAPTER XVI

HOST-PARASITE RELATIONS AMONG TRICHOMONADS

By

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INTRODUCTION

Three types of *Trichomonas,* one with three anterior flagella, one with four anterior flagella, and one with five anterior flagella, have been reported from man. Of these, the type with four anterior flagella, or *Trichomonas hominis,* is the most common.

Many of the lower mammals have also been shown to harbor trichomonads, and these may be roughly divided into two main types morphologically. One is massive in structure with a heavy axostyle, pronounced parabasal body, prominent undulating membrane, and forms cysts, *e.g., Trichomonas muris* of the white rat, and *Trichomonas caviæ* of the guinea-pig. This variety may, for convenience, be designated as the *Trichomonas muris* type. It has never been reported from man and has not been transferred to kittens, though one of the smaller trichomonads of the rat, either *T. parva* or *T. minuta* has (see Kessel, 1928*c*). It accordingly appears that this type in addition to possessing a characteristic morphology may possess certain definite cultural and host restrictions. One would therefore expect transmission experiments with this first type to be most successful between those hosts that harbor morphologically similar forms, *i.e.,* between the guinea-pig and the rat.

The other type exhibits a much more delicate gross appearance, the axostyle is much lighter in structure, the parabasal body is difficult to detect, and the undulating membrane is much less prominent. This general type is exhibited in the three trichomonads found in man, the common *Trichomonas* of the monkey, Brumpt (1909*b*), Prowazek (1912), Kessel (1928*d*), and of the domestic pig, Kessel (1928*a*), *Pentatrichomonas* (Wenrich and Yanoff, 1927) of the white rat, and the *Trichomonas* reported from kittens by da Cuhna and Muniz (1922), Brumpt (1925), Tanabe (1926), and Kessel (1926 and 1928*c*), and by Brumpt (1925) from the dog. This type has not been known to produce cysts. It may be designated as the human *Trichomonas* type.

INFECTION EXPERIMENTS

Attempts to infect lower mammals with *Trichomonas* of man have met with varying degrees of success, some workers recording positive results, and some negative. The positive reports of some of the earlier investigators were made without their being certain that the experimental animals with which they worked were actually negative for *Trichomonas* prior to the experiment. Consequently the negative results of Pringault (1920) in attempting to infect the rat, the rabbit, the guinea-pig, and the cat, and of Hogue (1922) to infect cats and rabbits were emphasized by Hegner (1927*c*) in his conclusion that "the host-parasite specificity of the human intestinal *Trichomonas* seems quite rigid."

It seems altogether probable that Hegner (1927*c*) was influenced in this conclusion concerning the rigidity of *Trichomonas* by his experiments with *Giardia*, most of his transmission experiments with *Giardia* having yielded negative results. He accordingly dismissed as valueless the earlier reports of positive transmission of *Trichomonas* and failed to attach significance in this relation to the work of Kessel (1926), in which kittens were experimentally infected with *Trichomonas* from man, *Macacus* monkeys, the white rat and the domestic pig, and also the work of Wenrich and Yanoff 1927), who infected the white rat with *Pentatrichomonas* of man. Knowles (1926) also reported infecting kittens with *Pentatrichomonas* of man.

As a result of the findings just mentioned, Kessel (1928*c*) concluded that *Trichomonas felis* da Cunha and Muniz (1922) was in all probability not a species of *Trichomonas* peculiar to the cat, but that *Trichomonas* infections in kittens might be acquired from a variety of different animals. He further stated that *Trichomonas* appears to be a parasite which is not restricted to a given host, but is one which will live equally well in several hosts. By analogy it seemed reasonable to conclude that in all probability *Trichomonas* infection in man might also be acquired from a variety of sources, and it seems likely that any animal which harbors the second general type of *Trichomonas* discussed above, in other words, any of the three types found in man, may serve as a reservoir host for human *Trichomonas* infections.

Hegner (1928*d*) was able to establish flagellate infections in young negative chicks. Among these were trichomonads from seven different species of hosts. After securing these positive results, Hegner (1928*d*) states, "There seems to be no doubt but that fowls are capable of transmitting certain human intestinal protozoa," and further suggests that several of the trichomonads established experimentally in fowls from different mammals "belong to a single species and not to separate species as regarded by many at the present time." He thus appears to have altered his earlier opinions regarding the rigidity of host-parasite relations, at least in so far as *Trichomonas* is concerned.

PROBLEMS AND METHODS

In addition to the trichomonads mentioned above, others have been reported from the armadillo by Fonseca (1915), by Braune (1913) from cattle, by Fantham (1920) from the horse, sheep and cattle. Trichomonads have also been found in birds, reptiles, amphibia, fish, leeches, mollusca and insects (see Wenyon, 1926).

Additional findings will undoubtedly occur and in studying the host-parasite relationships of this group, many more transmission experiments should be attempted. It would be expected that transmission will be easiest in the groups most closely related, although Hegner's results with fowls indicate a very wide range of transmission with this parasite, since he has already accomplished transmission between hosts belonging to two different classes of vertebrates.

The experimental method is the only method to employ in ascertaining these facts, and the experiments must be done with carefully selected negative animals. The best method to employ in selecting animals negative for *Trichomonas* is to culture freshly passed feces, collected by enema or following a purge, in the Locke-egg-serum medium, and examine for *Trichomonas*. This flagellate grows the most readily *in vitro* of any of the intestinal protozoa, and examination by this method (see Hegner and Becker, 1922) may be accepted as yielding most accurate results.

Negative animals selected by this method may be given *Trichomonas* by mouth or by rectum, the oral route being the more suitable in attempting to determine natural transmission.

Trichomonads to be experimentally transferred may be administered to the experimental animals with greatest facility by means of a stomach tube, a small French catheter attached to a Luer syringe being the most convenient. They may be given either in dilute freshly passed feces, or in culture serum in which they have been cultivated *in vitro*.

PATHOGENICITY

The question of the pathogenicity of *Trichomonas* is one that should be considered more carefully in the future than it has been in the past. Clinicians hold two opinions on this subject, one group maintaining that *Trichomonas* may produce diarrheic symptoms and the other that the *Trichomonas* is nothing more than a commensal that thrives well under diarrheic conditions. In experimental transmission work with animals, valuable data on the effect of *Trichomonas* on different animals may be procured. Kessel (1928*c*) records the presence of a diphtheritic colitis in kittens which appeared to be due to *Trichomonas* infection, and figures the invasion of *Trichomonas* into the gut tissue in a few instances. He has also recently observed a human autopsy in which the upper colon and cecum of a case positive for *Trichomonas* exhibited the same characteristic necrosis and diphtheritic colitis that he described in kittens. However, there was no invasion of the tissue by the trichomonads. Wenyon (1926) figures invasion of *Trichomonas* into human tissue taken at autopsy but was uncertain whether or not the invasion was due to post-mortem change. He states, however, that "guinea-pigs which are commonly infected with *Trichomonas caviæ* often show ulceration of the large intestine and cæcum with definite invasion of the tissue by the flagellate in perfectly fresh material." More extensive observations on the effect of the presence of *Trichomonas* in the intestine should be procured, and much important information may be obtained while transmission problems are in progress. In such instances it is imperative that the presence of other parasites or bacteria that might produce pathogenic symptoms in the experimental animals be ruled out by examination and culture.

CHAPTER XVII

INTESTINAL FLAGELLATES IN TISSUE CULTURE

By

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It has always been a question whether or not the intestinal flagellates are pathogenic. Some workers believe that they give off substances such as toxins and enzymes which are injurious to the tissues of the host. Other workers think that they cause mechanical injury to the cells of the tissues. It has been our hope to throw some light on these problems by combining the use of cultures of intestinal flagellates with tissues grown *in vitro*. If one can grow the tissue cells of the host outside of the body and then inoculate them with protozoa which are parasitic in that host one should be able to observe the effect of the parasite on the host and also to make a comparative study of the host and protozoan cells. This method involves the culturing of the protozoa so as to have large quantities of them when needed for the experiment and also the growing of various tissues from different animals in media in which the protozoa can live.

During the last few years the writer has been working along these lines, studying the mechanical effect of *Trichomonas* on the intestinal cells of chick embryos (Hogue, 1928) and making a comparative study of tissue cells and protozoan cells (Hogue, 1922*a*). So far the work is in its early stages and the problems open to investigators are numerous and alluring.

The cultivation of intestinal flagellates has been carried on by many investigators. Boeck (1921) cultured *Chilomastix mesnili* very successfully in a medium containing one part of human blood serum to four parts of Locke's solution.

Embadomonas intestinalis (Waskia intestinalis) was grown by the writer (Hogue, 1921*a*) on ovomucoid, Locke-Egg, ox bile medium and on Boeck's medium of Locke with a few drops of sheep or human serum. Ovomucoid medium is made in the following way: the white of one hen's egg is shaken up with glass beads. To this is added 100 c.c. of 0.7% sodium chloride solution. This is cooked for half an hour in a glass flask over a hot water bath and kept in constant motion. It is then filtered through cotton with a suction pump and about six cubic centimeters of the filtrate put in small test tubes. These are autoclaved for fifteen minutes at fifteen pounds pressure.

So far the different species of *Giardia* have not been grown outside of their hosts.

Trichomonas hominis has been grown by many workers, Lynch (1915), Hogue (1921*b*, 1922*b*), Cleveland (1925), and others. The chief things necessary for the growth of this flagellate seem to be a salt solution with some protein which may be either the white of hen's eggs or the serum of man, pig, or sheep. Both ovomucoid and sodium chloride sheep serum water have been successfully used by the writer. The latter is made in the following way: to a flask containing 100 cubic centimeters of 0.85% sodium chloride solution which has been sterilized in the autoclave add ten to fifteen cubic centimeters of sterile sheep serum water. The serum water is prepared by diluting one part of sheep serum with three parts of distilled water and sterilizing in the autoclave or Arnold sterilizer.

The pH should be somewhere between 6.8 and 8.4, though *Trichomonas* lives longest at pH 7.2-7.4 (Hogue, 1922*b*). The temperature of the incubator should be near that of the host, though most forms of protozoa will grow well at a temperature a few degrees below that of their host.

The trichomonads which have been used by the writer were obtained (1) from the mouth of man *(T. buccalis)* by rubbing sterile swabs along the teeth close to the gums and culturing the material obtained in sodium chloride sheep serum water (Hogue, 1926); (2) from human feces *(T. hominis* and *Pentatrichomonas ardin-delteili)*, from white rat feces *(T. muris)*, and from chicken feces *(T. gallinarum)* by putting pieces of feces the size of a small pea in test tubes containing six cubic centimeters of sodium chloride sheep serum water; (3) from the vagina *(T. vaginalis)* using sterile swabs and culturing in sodium chloride sheep serum water.

The protozoa were grown in large numbers together with many bacteria. The cultures were then diluted with sterile medium and the individual trichomonads were picked up by means of a Barber pipette, taking as few bacteria as possible. These were then introduced into a culture of fibroblasts, epithelium, mesothelium and sympathetic nerve fibers, grown from the stomach or intestine of a chick embryo seven or eight days old.

Since the above experiments were performed Cleveland has reported the obtaining of cultures of *Trichomonas* free from bacteria. These would be admirable for introducing into the tissue cultures. By using these the effect of the protozoa alone could be observed. In the writer's work so far reported only the mechanical effect of the protozoa could be studied, as a few bacteria were always present and the effect of their products on the tissue cells could not be distinguished from the effect of the protozoan products.

There are two general methods of growing tissues outside of the body; one the Harrison-Carrel method in which plasma and embryonic juices are used as a nutrient medium; the other the Lewis method where Locke-Lewis is used. The Lewis method is better fitted for these problems as it offers a liquid medium for the protozoa, whereas the plasma soon coagulates and becomes too dense for the protozoa to move about in.

For the Locke-Lewis (Lewis, 1924) chicken bouillon is very carefully made up and adjusted to pH 6.4-6.6. The Locke solution has the following formula: $NaCl$ 0.9%, $CaCl_2$ 0.025%, KCl 0.042%, $NaHCO_3$ 0.02%. In making the Locke-Lewis one uses eighty-five per cent of Locke solution, fifteen per cent of chicken bouillon and 0.25 to one per cent of dextrose. This is sterilized in the autoclave for fifteen minutes at fifteen pounds pressure.

Chick embryos from six to nine days old are used for the experiments. All the instruments, Petri dishes and coverslips are sterile. The eggs are opened at the broad end and the embryo removed with forceps or a spatula and placed in a small Petri dish containing Locke-Lewis which has been slightly warmed. Here the embryonic membranes are removed, the chick opened and the organs to be used in the experiments are taken out and placed in other small Petri dishes containing warmed Locke-Lewis in which they are cut into small pieces about 0.5 mm. long. With a sterile pipette two or three of these pieces with a small drop of the Locke-Lewis are placed on a coverglass which has been carefully cleaned and flamed. This is then inverted over the hollow of a depression slide which has previously been ringed with salvoline. This ring of salvoline seals the culture and also raises it well above the depression of the slide. The cultures are then incubated at 35°-37° C. for two or three days. By this time a good growth of cells has appeared from the explanted tissue and the cultures are ready for the introduction of the protozoa.

Sometimes the protozoa die soon after being introduced into the hanging drop of the tissue culture. The drop must not be too large or else the protozoa will remain in its lower part and never come in contact with the tissue cells which are in the upper part of the drop adhering to the coverslip. On the other hand, if the drop is too shallow or if the protozoa wander to the edge of it, they soon die. This may be because substances in the medium which are injurious to the protozoa collect on the surface or at the edge of the drop. It is quite certain that it is not due to the accumulation of carbon dioxide and waste products nor to the lack of oxygen, for when the protozoa were put on an ordinary slide and the coverglass sealed with salvoline they lived at least twenty-four hours and in one case they were found alive on the third day. The number had increased, showing that they had divided.

The problems open for solution by this method are unlimited since it appears that nearly all sterile tissues whether the animal be embryonic or adult can be cultured by the careful, resourceful worker. Barta and Petrovits (1928) have recently grown forty different sterile tissues from adult rabbits (six to twelve months old). Since most animals have parasites the problems are numerous. The important things to do are:

(1) to get pure cultures of protozoa,

(2) to grow the tissue from the host *in vitro,*

(3) to introduce the protozoa into the tissue culture and watch, hoping that the protozoa will be able to live in the tissue culture.

CHAPTER XVIII

INTESTINAL SARCODINA IN GENERAL

By

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Although the members of four of the orders belonging to the class sarcodina are almost all free living, a large number of species of amœbæ occur in the fifth order, the amœbida. General surveys of the class are to be found in Calkins' *Biology of the Protozoa,* pp. 313-341, in Wenyon's *Protozoology,* pp. 160-267, and in Hegner and Taliaferro's *Human Protozoology,* pp. 19-20. Calkins also gives a key to the genera of the sarcodina on pages 341-362. Calkins' account is largely devoted to free-living species whereas Wenyon and Hegner and Taliaferro emphasize parasitic species.

The order heliozoa contains protozoa commonly known as sun animalcules, most of which live in fresh water. Parasitic species, however, occur in several genera, for example, *Vampyrella* which is parasitic on algæ and *Nuclearia* which is parasitic in both algæ and protozoa. To the order radiolaria belong large numbers of marine protozoa that possess shells. Many of them have in their protoplasm vegetable organisms about fifteen microns in diameter with a cellulose wall. These are known as zooxanthellæ which are supposed to be symbiotic. A number of common fresh-water protozoa, such as *Arcella* and *Difflugia,* occur in the order foraminifera; others, especially the most complicated species, are to be found in salt water. Species belonging to several genera have been cultivated from the feces of man, horse and pig. Bêlâr (1921) recognizes six species in the genus *Chlamydophrys. C. stercorea* appears to be the most common. To obtain organisms of this type one should place pig's feces in a Petri dish in a moist chamber for a few days. The protozoa belonging to the order MYCETOZOA are characterized by a plasmodial adult stage. They are terrestrial protozoa living on decaying wood and leaves in moist places.

The order AMŒBIDA includes four families each of which contains parasitic and coprozoic species. In the family AMŒBIDÆ are large numbers of intestinal amœbæ including species that live in man as well as those living in lower animals. Every species of animal seems to be parasitized by one or more different species of amœbæ. These will be considered more in detail in other chapters in this book. Among the AMŒBIDÆ that are coprozoic is the species *Hartmannella hyalina*. This species has been cultivated from feces of man and other animals. Another species, one containing two nuclei, that has been found in human feces and that of certain other animals is *Sappinia diploidea*. The genus *Vahlkampfia* includes a number of species in the life-cycle of which no flagellate stage occurs. Members of this genus have been recovered from the intestine of a number of different types of animals and cultivated from the feces of man and other animals. A species that can readily be obtained for study is *V. patuxent* from the stomach of oysters, which has been studied in detail by Hogue (1921).

Amœbæ of the genus *Endamœba* are most frequent in the large intestine of man and other animals. Certain species, however, have been reported from the mouth and one species, *Endamœba histolytica*, appears to be able to live in almost any part of the human body. Among the species that can be obtained easily from animals are *E. blattæ* from the cockroach (Mercier, 1910; Lucas, 1927), *E. ranarum* from frogs, *E. muris* from the rat (Kessel, 1923, 1924) and various other species from insects, amphibia, reptiles and domesticated animals. A number of these have been cultivated, such as *E. barreti* from the tortoise (Barret and Smith, 1923) and *E. ranarum* from the frog (Barret and Smith, 1926), from cold-blooded animals, as well as *E. histolytica* and other species from warm-blooded animals. Cysts of certain of these species appear in cultures and methods of hatching them have been devised, making it possible to carry out various types of experiments with these organisms free from bacteria.

Since the discovery of *Endolimax nana* by Wenyon and O'Connor (1917) several new species belonging to this genus have been described. Apparently these organisms are so small that they are ordinarily overlooked. Monkeys, rats, fowls, lizards and frogs are known to be infected with species belonging to this genus. The writer has recently obtained species from ducks, guinea fowls and sheep by injecting material from the ceca of these animals into the rectum of parasite-free chicks. In the ceca of chicks *Endolimax* multiplies very rapidly and within a few days enormous numbers of specimens are available for study.

Not so many species of *Iodamœba* are known to occur in animals. Specimens of this genus have been recorded from monkeys and pigs. It seems probable that other animals are also infected with their own peculiar species of this genus. Thus far the only species of *Dientamœba* reported is *Dientamœba fragilis* which occurs in man. It has been suggested that this may be a species normally present in some other animal and only rarely a parasite of man since it has been reported from human beings in only a small number of cases.

To the family DIMASTIGAMŒBIDÆ belongs the species *Dimastigamœba gruberi* which is a coprozoic form that may be obtained without difficulty from the feces of man and other animals. It has a flagellate stage in its life-cycle and represents a species that might be considered either an amœba or a flagellate. *Mastigina hylæ* is a species that possesses a short flagellum. It occurs in the rectum of frogs and has recently been studied by Becker (1925, 1928). Another species, *Mastigina bovis,* has been reported from the stomach of cattle.

CHAPTER XIX

THE SPECIES OF HUMAN AMŒBÆ

By
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INTRODUCTION

The *amœbæ* of the human digestive tract offer an attractive field for biological research, as do also those of other mammals. Those of man are perhaps better known than are the amœbæ of any other vertebrate, but they still offer for this reason many attractive opportunities for research along new lines. The fields which have been opened by investigations to date may·be grouped according to the avenues of approach and the results aimed at, in the categories of morphological, developmental, faunistic, ecological, serological and immunological, diagnostic, medical, therapeutic, sanitary, and preventive.

The morphology of the amœbæ of man is but partially known. Published accounts and figures of most of the species are incomplete, often include aberrant or inadequately interpreted phases, and have been made generally with little reference to the particular environmental conditions under which their pseudopodia, cell contents and nuclear structures have been investigated. A dynamic morphology of some one of the several species found in man would be a distinct addition to our knowledge and also of assistance in interpreting the structural features found in the other species.

The particular morphological problems awaiting solution are mainly cytological, such as the comparative study of the mitochondria and their relation (if any) to the chromatoidal bodies; the search for a Golgi apparatus; and the critical analysis of the different so-called chromatins of the nucleus by the Fuelgen method, with a view to the distinctions between the peripheral and karyosomal chromatin and their changes under different conditions of metabolism and in mitosis. In addition, the chromosome number of all of the species should be completed, the chromosome cycle more fully elaborated with the aid of the Fuelgen method, and the critical value of the method itself should be verified for the PROTOZOA generally.

The morphology of the pseudopodia of the various species should be subjected to comparative study under experimental control with special reference to temperature, hydrogen-ion concentration, and a possible metabolic cycle in connection with mitosis.

The question as to races within the species based upon size as indicated by Dobell and others of the English school, should be subjected not only to statistical study by measurement, but to refined morphological comparisons, to continued study of individual cases of infection by a particular race over a period of time under varying conditions of diet and also in culture under varying conditions of media, temperature and hydrogen-ion concentration.

From the standpoint of development there should be a continued statistical study of an infection in man and in some amenable culture mammal in which encystment occurs, such as *Macacus,* under constant conditions of diet to determine whether or not there is any significant order in the fluctuations in number of the parasites and whether or not there are any critical moments in such cycles which might lead to the detection of, or to the increase of an improbability of sexual reproduction. A comparable study should also be made of the several species in culture, with a view to finding out the cytological and physiological interpretation of the fusions of active individuals which we have observed in *Endamœba gingivalis* and in two other species. In the developmental cycle both in feces and in cultures much remains to be done in establishing a timed chronology of the changes in the cyst and correlating this with the segregation and reduction of the glycogen and the formation, aggregation, and disappearance of the chromatoidal bodies with relation to external and internal conditions.

The possibility of a cycle within the bowel independent of discharge in the feces and of a cycle in the tissues in the case of *Endamœba dysenteriæ* should be investigated.

The faunistic aspects of the amœbæ of the human digestive tract are difficult of analysis, because of the movements of human population, and the practical difficulties of securing comparable data for the incidence of amœbic infections. All opportunities to make surveys of infections of any native groups more or less isolated from foreign contacts, should be seized upon and adequate material in the form of permanent preparations secured and the species determined with great care.

The indications from available statistics of infection point to the conclusion that these amœbic infections of man are age-old and race-wide and have few if any geographical or racial limitations. Confirming data are needed on this and especially from primitive peoples in Australia and Africa, such as the negrito stocks and the pygmies. It is possible that some significant variations in the incidence of infections by the different species of amœba may be found between the different geographical regions and different racial stocks. In any event, marked differences in the relative frequencies of infections, in the relative abundance of individuals, and in the incidence of multiple infections may be expected to be found in different ages, social groups, occupations, races and regions, and correlations with sanitary conditions and social customs will be brought to light by comparative surveys of these parasitic infections.

The ecological relations of these infections are but little known. Nothing is known as to how far, if at all, the different chemical elements of the diet, or particular types of food, favor, impede or inhibit the growth of these amœbæ *en masse* or differentially. The relations of the other animal intestinal parasites, of previous amœbic infections, of the sequence in the acquirement of infections, of reinfections, of the age at which they are acquired, of the accompanying bacterial complex of the feces, or of the particular species of bacteria and yeasts, to the acquirement, persistence, flare, or inhibition of the individual species of amœbæ are wholly unknown. Susceptibility of different individuals among culture animals should be tested, and the effect of varying numbers of cysts in establishing infections in new hosts or increasing infections in old hosts should be tried out. Comparable tests with material from cultures will be instructive. Changes in infectivity as a result of culture should be investigated.

Theories as to the phylogenetic relations of the amœbæ of man can be discussed only when a fuller knowledge of the amœbic infections of the mammals as a whole are better known and when host-parasite transfers have been studied more extensively, host specificity tested out more fully experimentally, and the border lines of mere survival of transferred parasites are more accurately determined. More must be known of the possibilities of transfer within new host species before much theorizing on phylogenetic trees of human parasites will have value.

It is especially desirable that our knowledge of amœbic infections in all of the primates be completed in order that some more evidence may be secured as to how far down the simian line the human infections may be traced. The distribution of the genera of human amœbæ in vertebrates as a whole is also of significance.

The infections in all of the various domesticated animals with which man has been associated in different parts of the world may prove to be instructive and significant as to the phylogeny of some of the human amœbæ.

The physiological problems awaiting investigation with human amœbæ are of necessity largely those accessible with amœbæ in culture, although certain problems are linked with the ecological ones, and others require correlated studies in some culture mammal. The metabolism of the various species in culture media should be investigated with reference to the nature of the chemical substances utilized, the changing requirements at different periods of the cycle, the effects of hydrogen-ion concentration and of oxygen tension upon metabolism, upon the cycle, and upon the rate of reproduction. The particular conditions which determine the location of amœbæ in restricted regions of the digestive tract, the effects of the different digestive secretions upon the amœbæ of the different species, and the factors in the stool or in the blood stream, or liver (if any), which favor encystment still await determination.

The effect of oxygen upon amœbæ in both the motile and encysted stages, in the intestine of culture mammals, and in culture media should be tested. The critical temperatures, both high and low, for each species in both the motile and the encysted stages are as yet imperfectly known.

The effects of the changes in the hydrogen-ion concentration within the limits of toleration for both motile and encysted stages, upon metabolism, and upon the shape and activity of the pseudopodia and the rate of movement invite investigation and a comparison among the different species both from stools and from cultures.

A particularly inviting field of investigation is the effect of radiant energy of different wave lengths throughout the various octaves available for use, alike on infections in culture animals, on amœbæ in cultures, and on encysted stages.

The biochemical problems have been noted in part in the discussion of the physiological ones. Specifically much may be done in a precise determination of the cultural requirements of the different species, the ranges of adaptation to changes in the amounts of organic and inorganic constituents of culture media, and the results of substitutions.

The cultural, serological, and immunological problems are closely interlocked and are interrelated with the physiological and biochemical. The prime desideratum is a method of establishing and of maintaining the intestinal amœbæ in pure culture, of freeing them with facility and certainty from bacteria and yeasts. When this is attained the field will be opened for investigations along serological lines, with precipitin and agglutination tests, with immunity and cross-immunity tests, with toxins and antibodies, and with intradermal tests for infection with *Endamœba dysenteriæ* and possibly for certain other species.

In the *diagnostic* field much remains to be done in determining the relative availability and practicability of the smear method of diagnosis by cysts in comparison with the culture method. In the latter method the difficulty of determining *with certainty* all of the species in the motile phases is very great and is, with our present knowledge, practically impossible.

Protozoölogical work is much needed in the medical and therapeutic field in a study of the morphological changes in the motile and encysted stages in stools and in cultures, resulting from the action of the various drugs and combinations of drugs used in the treatment of amœbiasis. The viability of both motile and encysted phases at successive stages of treatment should be checked by cultural methods. Correlations should be made between the various methods of treatment at different stages on the one hand and the morphological and functional changes in the parasite on the other. Control experiments with the drugs *in vitro* on cultures should also be made.

The effects of the treatments used against *Endamœba dysenteriæ* upon the other amœbæ of man should also be examined by comparable methods.

In the matter of tissue penetration by *Endamœba dysenteriœ, Councilmania lafleuri* and *C. dissimilis* much work is needed both on culture mammals and on man, in cases running an acute and also on those running a chronic course of amœbiasis. The blood-stream, the lymphatics, the spleen, lung, liver and gall bladder, and capillary networks generally should be investigated, for evidences of amœbic invasion, especially in cases of fatal, acute amœbiasis. Appendices removed in cases of chronic appendicitis should also be examined for evidences of amœbic invasion, with controls of stool examination for several weeks before operation.

The sanitary and preventive aspects of amœbic infections in man and domesticated animals associated with man have intimate relations to certain protozoölogical problems which center about host-parasite relations, the viability of cysts under different conditions in stools, and in respect to light, drying up of the stool, survival in dust, survival in and transit through the digestive tract of the fly and of other coprophile insects.

The extent to which transfers of the amœbic infections of man can be made into domesticated animals should be determined and distinctions established between the temporary maintenance of an infection, and its permanent continuance in a given new host and its transfer thence to other individuals of the new host species. The domesticated and other animals associated with man which should be tested are the pig, cat, dog, mouse, rabbit, and the domestic fowl and other barnyard birds.

CHAPTER XX

THE CULTIVATION OF *ENDAMŒBA HISTOLYTICA*

By

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Since the discovery of *Endamœba histolytica* by Loesch, in 1875, many investigators have claimed to have been successful in cultivating this parasite but it is only recently that success has been achieved in this direction. Until 1918, it is safe to say that the cultures of amœbæ obtained from human feces and supposed to be those of *E. histolytica* were really those of free-living species which were confused with the parasitic amœbæ of man and other animals. This mistake was made by Kartulis (1890), Musgrave and Clegg (1904), Walker (1908), Williams (1911) and many others, and it is now generally admitted that none of the amœbæ cultivated by these observers were *E. histolytica* but were free-living amœbæ that had contaminated the material cultured or the culture media. It was not until Walker, in 1911, as the result of a careful study of cultural amœbæ, had proven them all to be free-living species and that it was impossible, with the methods then available, to cultivate *E. histolytica*, that it was generally recognized that these cultural amœbæ had nothing in common with *E. histolytica*, or other parasitic amœbæ, and that none of the latter had been cultivated.

Walker's work confirmed that of the writer, in this respect, and it may be stated with truth that all of the reports of the successful cultivation of *E. histolytica* prior to 1918 are inaccurate, being based upon erroneous observations and that the amœbæ cultivated prior to that time were, in all probability, free-living species belonging to the genera *Amœba, Vahlkampfia,* or *Hartmanella.*

In 1918, Cutler claimed to have cultivated *E. histolytica* upon a blood-clot medium and upon an egg medium. The cultures were found to grow best at temperatures between 28° and 30° C. He obtained cultures from six stools in cases of acute amœbic dysentery and was successful, in one instance, in keeping the cultures going for several weeks, subcultures being made every day. The reader is referred to Cutler's paper for details regarding his work and, although Dobell (1919), because he was unable to confirm Cutler's results, does not believe he actually cultivated *E. histolytica,* there is very good reason to believe that he was successful in this respect. Wenyon (1926) and Knowles (1928) both state that Cutler was probably successful in cultivating this parasite, so that it is most probable that to Cutler belongs the credit of having first obtained *E. histolytica* in cultures and of having maintained such cultures for several weeks.

While there may still be some doubt of Cutler's success in the cultivation of *E. histolytica* there is none at all in that of Boeck and Drbohlav, who, in 1925, published their results in this respect and gave to the protozoölogist a simple and successful method of cultivation of this important parasite. Their work proved conclusively that this parasite can be cultivated and that the amœbæ in the cultures are capable of producing typical amœbic dysentery in kittens accompanied by the characteristic lesions of the infection. These investigators cultivated this amœba from the feces of two patients with amœbic dysentery whose stools were free from other intestinal protozoa. The first successful culture was obtained in Locke-Egg-Serum medium while cultivating the stools of one of the patients for other intestinal protozoa. This strain of the parasite was continued in cultures for over eight months and through 152 subcultures.

The work of Boeck and Drbohlav was soon confirmed by many protozoölogists and there have been many modifications of their culture media and technique. For the cultivation of *E. histolytica* many media have been recommended and the most useful and important of these will now be considered.

CULTURE MEDIA FOR ENDAMŒBA HISTOLYTICA

Boeck and Drbohlav's Media. The two following media have been found efficient in the cultivation of this parasite by Boeck and Drbohlav (1925):

MEDIUM No. 1. *Locke-Egg-Serum,* or *L.E.S. Medium.* Four eggs are washed, brushed with alcohol and broken into a sterile flask containing glass beads. Fifty cubic centimeters of Locke's solution (see below) are then added and the mixture broken up by shaking. Test tubes are then filled with sufficient of the mixture to produce slants of from one to one and one-half inches in length upon coagulating by heat. The tubes are then slanted in an inspissator and heated at 70° C. until the egg mixture is solidified. They are then autoclaved at fifteen pounds pressure for twenty minutes. The tubes are then covered to a depth of one centimeter above the egg slant with a mixture composed of eight parts of sterile Locke's solution and one part of sterile inactivated human blood serum, and incubated to determine sterility.

MEDIUM NO. 2. *Locke-Egg-Albumen*, or *L.E.A. Medium*. Drbohlav modified the above medium by using crystallized egg albumen instead of human blood serum. A one per cent solution of the crystallized egg albumen in Locke's solution was employed and this was sterilized by passage through a Berkefeld filter and then added to the tubes containing the egg slants as described above for the L.E.S. medium.

The initial reaction of these media varied from pH 7.2 to pH 7.8 and needed no adjustment.

The Locke solution used had the following formula:

NaCl	9.0 gms.
$CaCl_2$	0.2 gm.
KCl	0.4 gm.
$NaHCO_3$	0.2 gm.
Glucose	2.5 gms.
Distilled water	1000.0 cc.

The solution is sterilized in the Arnold or the autoclave. The human blood serum must be passed through a Berkefeld filter after adding the Locke solution, and sometimes a second filtration is necessary.

E. histolytica may be cultivated without difficulty upon either of these media and subcultures should be made every other day from the sediment at the bottom of the tubes, obtained by a sterile pipette. The most favorable temperature for incubation was found to be 37° C.

Kofoid and Wagener's Medium (1925).

These investigators have modified the L.E.S. medium of Boeck and Drbohlav by adding to the egg slant ten cubic centimeters of Locke's solution containing 0.5 cubic centimeter of defibrinated rabbit blood. They state that this medium is not as favorable to the growth of bacteria as the Boeck and Drbohlav media, thus favoring the growth of the amœbæ, and that transfers should be made every forty-eight hours.

Dobell and Laidlaw's (1926) Media. These investigators recommend the two following media in the cultivation of *E. histolytica:*

MEDIUM NO. 1. An egg-serum medium made as recommended by Boeck and Drbohlav but with Ringer's solution instead of Locke's solution. Ringer's solution contains 9 grams of sodium chloride, 0.2 gram of calcium chloride, and 0.2 gram potassium chloride to the litre of distilled water. The covering fluid for the egg slant is egg albumin or inactivated horse serum diluted one part to eight with the Ringer solution. Just before making the culture for amœbæ a small amount of rice starch which has been sterilized is added to the medium under aseptic precautions. The rice starch is sterilized by heating in a dry air oven at 180° C. for one hour, about 2.5 grams of the starch being placed in a small glass tube. If egg albumin is used instead of horse serum the whites of four eggs are mixed with a litre of Ringer solution and the whole sterilized by filtration. No adjustment of the reaction of this medium is necessary.

MEDIUM No. 2. This medium is similar to MEDIUM No. 1 except that undiluted horse serum is used for the slants instead of egg and Ringer solution. The horse serum is sterilized by filtration, tubed, slanted and inspissated at 80° C. for one hour or an hour and ten minutes and no longer. The tubes are then cooled, incubated for sterility, and then the slant covered with egg albumen solution or serum prepared as in MEDIUM No. 1. Starch is added at the time of culture as described above. No adjustment of the reaction of this medium is necessary.

Dobell claims that *E. histolytica* grows more abundantly and lives longer upon these media, especially the horse-serum-egg-albumen-starch medium, than upon the media of Boeck and Drbohlav, sometimes living for as long as two weeks and that subculture is not necessary more often than once a week. The starch also prevents the development of *Blastocystis hominis* and replaces the dextrose used in the Boeck and Drbohlav media.

Craig's Media. In the writer's observations upon the cultivation of *E. histolytica,* the solid egg or serum slants used in the media just mentioned have not been found to be necessary and the belief of Kofoid and Wagener (1925) that either the coagulated egg or blood contained in blood agar slants is required for at least a part of the food supply of this amœba, has been found to be erroneous. In 1926, the writer published a simplified method for the cultivation of this parasite in which no solid substratum was used, and in a later paper (1926*a*) published still more simple methods for cultivating the organism. The observations noted demonstrated that none of the constituents of either Locke or Ringer's solution, except possibly the sodium chloride content, are absolutely essential for the cultivation of *E. histolytica,* as a simple mixture of normal salt solution and inactivated human blood serum is a suitable culture medium for this parasite, although it cannot be maintained in this simple medium indefinitely, as it can in most of the other media that have been evolved. The following media have all been found efficient in culturing *E. histolytica* in the writer's hands:

MEDIUM No. 1. *Locke-Serum Medium.* This medium consists of a mixture of Locke's solution and either inactivated human, horse, or rabbit blood serum. Inactivated human blood serum has been found to give the best results and this serum should not be over forty-eight hours old when used.

The Locke's solution used has the following formula:

Sodium chloride	9.00 gms.
Calcium chloride	0.24 gm.
Potassium chloride	0.42 gm.
Sodium bicarbonate	0.20 gm.
Dextrose	2.50 gms.
Distilled water	1000.00 c.c.

This solution is filtered and autoclaved at fifteen pounds pressure for fifteen minutes, and allowed to cool. There is then added one part of inactivated human blood serum to each seven parts of the Locke solution used. After adding the blood serum the mixture is filtered through a Berkefeld filter and it is sometimes necessary to filter through two or more candles before the mixture comes through perfectly clear. After the filtration the medium is tubed, placing about ten cubic centimeters in each tube, and incubated for twenty-four or thirty-six hours to determine sterility. If found sterile the tubes should be kept in an incubator at 37° C. until used. The reaction of the medium does not need adjusting. The addition of a little rice starch, as recommended in the description of the media of Dobell and Laidlaw, adds to the efficiency of this medium. A small amount of the sterilized starch may be added to the culture media at the time of the inoculation of the material to be cultured. The blood serum is inactivated by heating at 56° C. for one-half hour.

MEDIUM No. 2. *Ringer-Serum Medium.* The Ringer solution used in this medium has the following formula:

Sodium chloride	8.00 gms.
Calcium chloride	0.20 gm.
Potassium chloride	0.20 gm.
Distilled water	1000.00 c.c.

This solution is filtered, sterilized, and to each seven parts of it is added one part of human blood serum inactivated by heating at 56° C. for one-half hour. After adding the serum the mixture is filtered through a Berkefeld filter, tubed, and kept in the incubator at 37° C. for twenty-four to forty-eight hours to determine sterility. The tubes should be stored in the incubator at this temperature until inoculated, and incubated at the same temperature.

In order to secure the best results with the two media transfers should be made every twenty-four to forty-eight hours. In these media *E. histolytica* may be maintained for periods as long as three months but not indefinitely.

MEDIUM NO. 3. *Normal-Salt-Serum Medium.* One part of inactivated human blood serum is added to seven parts of normal salt solution (0.85%), filtered through a Berkefeld filter, tubed, and incubated at 37° C. to determine sterility. If sterile the tubes are kept in the incubator at this temperature until inoculated and incubated at the same temperature.

In this exceedingly simple medium *E. histolytica* has been found to grow well for a limited period of time, cultures having been maintained in this laboratory for over two months, transfers being made daily or every two days. It is not as satisfactory a medium for the indefinite maintenance of cultures as the modified Locke-Egg-Serum medium or the Locke-Serum medium.

Choice of Culture Method. For *ordinary diagnostic work, i.e.,* the demonstration of *E. histolytica* in the feces, the writer prefers the Locke-Serum medium, although practically as good results are obtained with the Locke-Egg-Serum medium of Boeck and Drbohlav, modified by the omission of dextrose, which is not necessary. The addition of rice starch is not necessary in ordinary diagnostic work, but this substance added to either medium increases their value if cultures are to be maintained for a long period of time. The simple Normal-Salt-Serum medium has given good results, in diagnostic work, in the hands of several observers, and Vogel (1928) and Kolle and Hetsch (1929) recommend this medium for the cultivation of this parasite. Magath and Ward (1928), at the Mayo Clinic, in a comparison of the Boeck-Drbohlav Locke-Egg-Serum medium and the Normal-Salt-Serum medium, in the cultivation of amœbæ from the stools, obtained better results with the latter medium. They obtained eighty-eight positive cultures from 521 stools, or 16.9%, with the Craig Normal-Salt-Serum medium, while with the Boeck and Drbohlav medium they obtained fifty-two positive cultures from 394 stools, or 14.7%. Of 115 stools cultured by both methods, the Normal-Salt-Serum medium gave twenty-three positive cultures, or 20%, while the Boeck-Drbohlav medium gave twenty positive cultures, or 17%.

For the *maintenance of cultures by transfer,* the writer prefers the Boeck-Drbohlav Locke-Egg-Serum medium, modified by the omission of dextrose, transfers being made every forty-eight hours. The addition of rice starch, as in the Dobell-Laidlaw media, is useful at times, if the amœbæ appear to be decreasing in number, but is not necessary as a routine procedure. Upon this medium we find that the parasite will live indefinitely, and we now have a strain in this laboratory that has been cultured for over three years and which still is normal in morphology and produces lesions in kittens.

Technique of Cultivation. Aseptic precautions are not absolutely necessary in the inoculation of the culture media for clinical diagnostic purposes, although it is best to observe such precautions, but in making transfers it is always best to observe ordinary asepsis in the technique. For diagnostic work a good-sized particle of the formed feces, or several large loopsful of the liquid or semi-liquid feces, is emulsified in the liquid portion of the medium, if slants are used, or in the Locke-Serum medium. If the feces are formed the inoculated material should be thoroughly broken up in the medium with a platinum loop. After inoculation the cultures are placed in an incubator at 37° C. for twenty-four hours and then a small amount of the sediment, taken from the culture with a one cubic centimeter pipette, having a rather large bore, should be placed upon a microscopic slide, covered with a cover-glass, and examined. If negative the culture should be replaced in the incubator and re-examined at the end of another twenty-four hours.

Some authorities have not secured good results in cultivating *E. histolytica* from the stools because they have employed ordinary bacteriological technique in the examination. The amount of feces inoculated into the media should be much larger than a platinum loopful, the usual amount of material used for inoculating culture media in bacteriological work, and it is absolutely useless to examine a loopful from the cultures and expect to find amœbæ. A one cubic centimeter pipette, with a good-sized bore, should always be used in securing the sediment from the cultures for examination, and at least one-tenth of a cubic centimeter should be removed and examined. The amœbæ are most numerous in the sediment at the bottom of the tubes and upon the surface of the egg slant at the bottom of the tubes, if the Locke-Egg-Serum medium is used. The amœbæ do not occur in the fluid portion of the culture much above the sediment.

It should also be remembered that the amœbæ never occur in the cultures in numbers comparable to bacteria in cultures and many fields of the microscope will usually have to be searched before a single amœba is encountered. This fact has caused inexperienced workers to regard cultures as negative which were actually positive and it should be the practice to examine at least three preparations from a culture before returning a negative report. In rich cultures the amœbæ seldom number more than two to four in one field of the microscope and many microscopic fields will not show any amœbæ. In cultures made directly from the feces it is rare to observe even two amœbæ in a microscopic field but in transfers the amœbæ are more numerous.

If the technique recommended be employed it will be found that cultures of the feces not infrequently give positive results in individuals in whom the direct microscopical examination was negative, and this is especially true if the feces are formed.

In order to maintain strains of *E. histolytica* in culture it is best to make subcultures every forty-eight hours and the modified Locke-Egg-Serum culture medium is perhaps most satisfactory for this purpose. Certain precautions are necessary in order to prevent the loss of the culture, the most important being the maintenance of the cultures at a temperature between 37° and 38° C., the prevention of bacterial contamination other than that originally present in the culture, and the use of a carefully made medium in which no variation occurs in the quantity of the constituents. It is especially important that the proper temperature be maintained, for this parasite is very sensitive to changes in temperature, especially to cold, and we have lost many strains by reason of the electric current of our incubators failing at times. It is also necessary to use aseptic precautions in making subcultures, for a change in the bacterial content of the cultures frequently results in the death of the amœbæ. Having obtained a strain of *E. histolytica* in culture it is best to prevent bacterial contamination with bacteria not present in the original culture, for the introduction of other bacteria frequently leads to the death of the amœbæ. In our laboratories the introduction of such foreign bacteria, through failure of asepsis in transferring cultures, has frequently led to the loss of the strain of the parasite under cultivation.

Care should also be taken to transfer a considerable amount of the sediment in the cultures as often the amœbæ are present in small numbers at certain intervals and unless this is done the strain will be lost. Use a one cubic centimeter pipette with a good-sized bore and transfer as much of the sediment as possible. In our experience the number of amœbæ vary considerably in the cultures and there is some indication of a more or less regular cycle in the growth of the organisms in culture so far as number is concerned. Upon certain days the amœbæ are so few that one almost believes the organisms to have perished but on succeeding days they will again become very numerous.

The Value of the Cultivation of E. histolytica *in Diagnosis.* In 1922, Hegner and Becker called attention to the value of cultures in the diagnosis of infestation of the intestine with flagellates, and, in 1926, St. John demonstrated the value of the cultivation of *E. histolytica* from the feces in the diagnosis of amœbiasis, obtaining a higher percentage of positive results by culture than by the direct microscopic examination of the feces. In 1927, the writer and St. John published the results of the examination of seventy-one individuals, using direct microscopical examination of the feces, cultural methods, and sedimentation tests, in comparison, one with another. The following tables illustrate the results obtained with these various methods.

TABLE 1. RESULTS WITH DIRECT MICROSCOPICAL EXAMINATION

Species of Amœba	Positive	Negative	Per Cent Positive
Endamœba histolytica	6	65	8.45
Endamœba coli	8	63	11.26
Endamœba nana	2	69	2.81
Iodamœba williamsi	3	68	4.21

TABLE 2. RESULTS WITH CULTURAL METHODS

Species of Amœba	Positive	Negative	Per Cent Positive
Endamœba histolytica	11	60	15.49
Endamœba coli	21	50	29.57
Endamœba nana	9	62	12.67
Iodamœba williamsi	4	67	5.63

TABLE 3. RESULTS WITH SEDIMENTATION TEST

Species of Amœba	Positive	Negative	Per Cent Positive
Endamœba histolytica	9	62	12.67
Endamœba coli	12	59	16.90
Endamœba nana	3	68	4.21
Iodamœba williamsi	3	68	4.21

While the number of individuals examined was small and the percentage of positive results must be interpreted with caution owing to this small number, it is very evident that the cultural method of examination of the feces gave a higher percentage of positive results for *Endamœba histolytica* than any other method, and these results have been confirmed by nearly four years' use of this method at the Army Medical Center, where the cultural method not infrequently picks up infections with this parasite that are missed by the ordinary microscopic examination of the stool.

Our observations also demonstrated that the Locke-Serum medium gave a higher percentage of positive results for *E. histolytica* than the modified Boeck and Drbohlav Locke-Egg-Serum medium. With the latter medium nine of the seventy-one individuals examined showed this parasite in the feces, or 12.67%, while with the Locke-Serum medium, eleven, or 15.49%, showed *E. histolytica* in the cultures.

As a result of nearly four years' use of the culture method in the examination of stools for *E. histolytica* the writer believes that this is the best method of making such an examination. It invariably results in the demonstration of the parasite in all cases in which it may be demonstrated by direct microscopic examination, and not infrequently it demonstrates the organism in cases in which the direct microscopic examination had resulted negatively. However, it is absolutely necessary that the culture media be prepared as described and that the technique of inoculating and examining the cultures noted in this contribution be followed. Time and again, the writer has found that investigators have failed to culture *E. histolytica* because bacteriological technique was followed in the examinations and inoculations, or other deviations occurred from the proper technique. Wherever the proper technique has been used the cultural examination of feces for this parasite has been attended with excellent results but in this, as in other laboratory procedures, experience is necessary, and this cannot be acquired without devoting much time and attention to the subject.

In conclusion, it may be stated that the cultivation of *Endamœba histolytica* has made possible great advances in our knowledge of the life-history and biology of this important parasite of man and has added a method of much diagnostic value to human medicine. It is undoubtedly true that it is only a question of time before a method of obtaining pure cultures of this parasite will be evolved and when this is accomplished further light will be thrown upon the life-history and biology of this and other organisms belonging to the AMŒBIDA.

CHAPTER XXI

SEROLOGICAL STUDIES WITH *ENDAMŒBA HISTOLYTICA*

By

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The biological activities of *Endamœba histolytica,* the cause of amœbic dysentery, have always excited the interest of the medical zoölogist, owing to the great importance of this parasite as a pathogenic organism living in man, and to the marked and characteristic lesions which it produces frequently in the tissues of its human host. For many years it has been known that this parasite possesses the property of phagocyting the red blood corpuscles of man and certain of the lower animals and of penetrating the tissues of the intestine, producing necrosis and the formation of ulcers and abscesses. It has also been found in other parts of the body, as the liver, the brain, the testicles, and the skin, where it produces abscesses of peculiar character; while in experimental animals, as the dog and cat, similar pathological lesions have been produced experimentally by numerous observers.

The theories regarding the biological activities of *E. histolytica* until quite recently were based entirely upon clinical and pathological observations, and this has been especially true of the serology of man and other animals infected with this parasite. This has been due to the fact that it was not until recently that cultures of this organism were available for use in experimental research, but the successful cultivation of *E. histolytica* by Boeck and Drbohlav (1925) and others has made possible more accurate observations regarding this subject.

In 1927, the author published observations demonstrating the presence in alcoholic extracts of cultures of *E. histolytica* of hemolytic and cytolytic substances. While the conception that a portion of the pathogenic action of this parasite upon the tissues of man and other animals was due to a toxin, or toxins, cytolytic in nature, secreted by the organism, has been held for many years by students of the subject, it was not until this demonstration that it was shown experimentally that such agents did exist in the parasite, and that alcoholic extracts contained these substances. The work referred to demonstrated that in alcoholic extracts of forty-eight-hour cultures of *E. histolytica*, grown upon the Locke-Egg-Serum medium of Boeck and Drbohlav (1925), modified by using human blood serum instead of horse serum, and in Locke-Serum medium (Craig, 1926), there is present a hemolysin capable of dissolving the red blood corpuscles of man, rabbits, and guinea-pigs. This hemolysin was found to be thermolabile, being destroyed when heated in a water bath at 60° C. and practically insoluble in normal saline although soluble in absolute alcohol. It was found not to be an exotoxin, as it could not be demonstrated in the fluid portion of the cultures, in cultures in which the amœbæ were dead, or in cultures over forty-eight hours old. The experiments demonstrated that the hemolysin was intimately connected with the living amœbæ as extracts of cultures containing only dead amœbæ did not contain the hemolysin.

These extracts of *Endamœba histolytica* also contained a cytolysin capable of dissolving the epithelial cells of the mucous membrance of the intestine of both man and cats, but neither the hemolysin nor cytolysin was specific for the cells of man, as already indicated, for the cytolysin dissolved the intestinal epithelium of both man and cats, while the hemolysin dissolved the red blood corpuscles of man, cats, and guinea-pigs. Bacteriolytic substances could not be demonstrated in the extracts, and this observation is significant in view of the well-known fact that *Endamœba histolytica* does not ingest bacteria under normal conditions in man although it does ingest and dissolve the red blood corpuscles of its host.

The observations mentioned, although of much interest from the standpoint of the etiology of the lesions characteristic of the invasion of the tissues of man and experimental animals by *Endamœba histolytica*, are not of as great theoretical and practical interest as the fact that these extracts, when used as antigens in a complement fixation test, have been found capable of demonstrating the presence of complement fixing bodies in the blood serum of individuals infected with this parasite, thus proving that antibodies are produced to this infection, and explaining the occurrence of many cases of infection with this parasite in which symptoms are absent or so mild as not to attract attention. This apparently specific reaction has been found useful in the diagnosis of infections with *E. histolytica* and it is probable that further work in perfecting the extracts used as antigens may render the complement fixation test for *E. histolytica* of general clinical value as a routine diagnostic test.

Historical. Before considering the subject of complement fixation a brief review of what has been accomplished in the serology of infections with *Endamœba histolytica* may be of interest.

Perhaps the first contribution of moment upon the serology of these infections was that of Izar (1914), who claimed to have obtained complement fixation reactions with the blood serum of five human infections with *E. histolytica* and the blood serum of three cats infected with this parasite, using as an antigen aqueous extracts of the feces of infected cats and the pus from a liver abscess in man. His work was repeated in 1920, by Hage, who was unable to confirm Izar's results, but the lack of confirmation may have been due to numerous factors, as variations in the technique, the amount of antigenic substance in the antigens employed by the two workers, and the character of the infection in the individuals tested. In the light of the work recorded in this contribution it is extremely doubtful if the antigens employed by Izar would prove of clinical value in the diagnosis of amœbic infections, but it is reasonable to believe that he did obtain complement fixation with the antigens that he used in his work.

More recently, Wagener (1924) has recorded her results in an attempt to devise a precipitin test for *E. histolytica* in experimentally infected kittens, using as an antigen scrapings of amœbic ulcers of the colon of the cat. This work was adequately controlled and resulted in the demonstration that in the blood serum of cats infected for a week, or longer, after the amœbæ were observed in the stools, there occurred antibodies which gave a positive precipitin reaction with such antigens and that the blood of normal cats, or cats infected for less than a week after the appearance of the amœbæ in the stools gave negative precipitin reactions.

The preparation of the antigen, the controls employed, and the technique of the tests are described in the paper by Wagener and the reader is referred to this for details. The results of the test as recorded by Wagener were as follows: seven cats were tested which had discharged *E. histolytica* in the stools for eight days, or longer, and these all gave strongly positive precipitin reactions up to one to four dilutions of the blood serum, while three cats with an infection lasting from six to eight days gave a weak reaction with undiluted blood serum. Six cats dying in less than six days after the development of dysentery gave negative precipitin reactions, as well as five normal cats. The bacterial controls of the antigen always gave negative precipitin reactions.

PERSONAL OBSERVATIONS

For a period of over three years I have been studying complement fixation in infections with *Endamœba histolytica* and have published preliminary papers upon the subject in 1927 and 1928. Since the publication of these papers additional data have been accumulated regarding the subject which will be noted in this contribution.

Technique. The general technique of the complement fixation test used in this work has been practically the same as the standard method for performing the complement fixation test for syphilis used in the laboratories of the Medical Department of the United States Army, which is fully described in my book entitled *The Wassermann Test.* The methods of preparing the reagents used, with the exception of the antigenic extracts, and their titration were like those used in the regular complement fixation test for syphilis, as used in the army, except that in titrating the antigenic extracts, owing to their weak antigenic power, it was necessary to use them without dilution. A human hemolytic system was used, the blood sera tested inactivated by heating in a water bath at 37° C. for one-half hour, and the quantities of blood sera and saline were identical with those used in the standard test for syphilis. The preliminary incubation with antigen and complement was one-half hour in the water bath at 37° C., and the incubation after adding the hemolytic amboceptor and blood suspension was one full hour in the water bath at 37° C. and at least two hours in the ice-box, at the end of which time the results were recorded. The ice-box incubation was very important, for generally hemolysis was far from complete in negative cases at the end of the one hour incubation in the water bath and did not become complete until the ice-box incubation was completed.

The antigenic extracts used in the tests were prepared by extracting with from seven to ten parts of absolute alcohol the sediment of from sixty to eighty or more cultures of *E. histolytica* grown upon the Locke-Egg-Serum medium of Boeck and Drbohlav (1925), modified by using inactivated human blood serum instead of horse serum. The cultures were forty-eight hours old and the extraction was carried out in the incubator at 37° C. for ten days, the mixture of alcohol and culture being thoroughly shaken several times a day during that time. After extraction the mixture was filtered and the filtrate used as an antigen in the tests. Owing to the weak antigenic value of the extracts it was necessary to use them undiluted and in the titration it has usually been found that the workable antigenic amount varied from 0.06 to 0.1 of one cubic centimeter. The latter amount was hemolytic if added to a blood suspension which did not contain blood serum but if the usual amount of blood serum was added this amount of antigenic extract was not hemolytic and could be used in the tests.

Owing to the fact that the antigenic extracts must be employed undiluted the range of the test was markedly restricted and great care must be used to see that all reagents were very accurately measured. Briefly stated, the technique of the test was as follows:

A double rack was employed, as in the Wassermann test, the anterior row of tubes containing the sera to be tested and the posterior the controls without the antigenic extract. In each tube there is placed 0.9 of one cubic centimeter of normal salt solution, double the amount of complement determined by titration as sufficient to cause hemolysis of the standard amount of blood suspension, and 0.1 of one cubic centimeter of the serum to be tested. To the anterior tube there is now added the unit of antigenic extract which has been determined by titration and the rack placed in the water bath at 37° C. for one-half hour. At the end of this time there is added to each tube 0.1 of one cubic centimeter of the blood suspension, and two units of anti-human hemolytic amboceptor, after which the rack is kept in the water bath at 37° C. for a full hour and is then kept in the ice-box for two hours longer, when the results of the test are recorded. It has been my practice to read the results again after incubation in the ice-box over night, as most strongly positive cases are still positive at the end of this time.

As controls alcoholic extracts of all the bacteria growing in the cultures with *E. histolytica*, both aërobic and anaërobic, have been prepared and used in the same manner as the antigenic extracts and have always given negative results in the test. Their use has now been abandoned because of the uniformly negative results obtained in a very large series of experiments and our experience has proven that, while desirable, these bacterial extracts are not necessary as controls in the practical application of the test.

In the experimental work the sera tested were taken at random from specimens submitted to the serological laboratory of the Army Medical School for the complement fixation test for syphilis and such sera are used continually as controls of the sera submitted by clinicians for the complement fixation test for *E. histolytica.*

After testing the blood sera, a specimen of feces is always requested from each patient tested, whether the result is positive or negative, and this is examined microscopically, culturally, and, if necessary, by the sedimentation test for the cysts of *E. histolytica.* Thus all of the results reported in this contribution have been checked by such examinations of the feces of the individual supplying the blood serum tested.

Results of the Complement Fixation Test. At the time of writing 570 blood sera, from as many individuals, have been tested, and the results checked by an examination of the feces. Of these sera, sixty-one or 10.7%, gave a positive reaction, and 509, or 89.2%, gave a negative reaction. Of the positive cases, fifty-three gave a four-plus reaction, and eight gave a three- or two-plus reaction. The large percentage of positive cases giving absolute inhibition of hemolysis, or a four-plus reaction, is noticeable with this test and renders it very easy to read and evaluate.

Of the sixty-one positive cases, a check of the feces for *Endamœba histolytica resulted in the demonstration of this parasite* in fifty-five individuals, or 90.1%. In six individuals we were unable to demonstrate this organism in the feces although in two of these there was a history of amœbic dysentery.

Of the sixty-one positive cases, eight were mixed infections with *Endamœba histolytica* and *Endamœba coli;* two mixed infections with *Endamœba histolytica* and *Endamœba nana;* two with *Endamœba histolytica, Endamœba nana,* and *Endamœba coli;* two with *Endamœba histolytica* and *Chilomastix mesnili;* and two with *Endamœba histolytica* and *Giardia intestinalis.* Forty-five of the positive cases showed a pure infection with *Endamœba histolytica.*

Clinically, over sixty-five per cent of the individuals giving a positive reaction presented symptoms that were probably caused by the parasite, while in the remainder, if symptoms had been present they were not noted by the patients. In other words, these latter individuals were so-called "healthy carriers" of *E. histolytica.*

The large percentage of positive reactors presenting symptoms is accounted for by the fact that they were mostly hospital patients suffering from gastro-intestinal symptoms or in hospital for other conditions, and many of them had a history of amœbic dysentery. In practically all of the patients presenting symptoms of the infection, who gave a positive reaction, proper treatment which caused a disappearance of the amœbæ from the stools resulted in the disappearance of the symptoms, and also in a disappearance of the positive reaction within from three to six weeks after treatment in all who were tested.

Of the 509 individuals whose blood sera gave a negative reaction with the complement fixation test, an examination of the feces resulted in the discovery of only five infections with *E. histolytica,* or about 0.9%. It is interesting to note that in these cases the symptoms of amœbic dysentery were very acute and amœbic abscess of the liver was present in one case. Among the 509 individuals giving a negative complement fixation test 144, or 28.2%, showed the presence in the feces of some other species of protozoa. There were sixty-seven individuals, or 13.2%, with *Endamœba coli* in the feces; forty-one, or about eight per cent, with *Endamœba nana* in the feces; three, or approximately one-half of one per cent, with *Iodamœba williamsi;* seventeen, or 3.3%, with *Chilomastix mesnili;* eleven, or 2.1%, with *Trichomonas hominis;* and five, or approximately one per cent, with *Giardia intestinalis* in the feces. There were eleven mixed infections with *Endamœba coli* and *Endamœba nana;* four with *Endamœba coli* and *Chilomastix mesnili;* three with *Endamœba coli* and *Giardia intestinalis,* and one with *Endamœba coli* and *Trichomonas hominis.* In 111 of the cases giving a negative complement fixation reaction, or 21.6%, some other species of amœba other than *Endamœba histolytica* was present, and from these results it is evident that infestation of the intestine by species of amœbæ other than *E. histolytica* or by flagellates does not cause a positive complement fixation reaction in blood sera with the antigenic extracts used in this series of experiments.

The individuals giving a negative complement fixation test were mostly in hospital and were suffering from a wide range of disease conditions, so that it is justifiable to state that disease conditions other than amœbic infection, with the possible exception of rare cases of syphilis, as will be noted later, do not give a positive reaction with this test.

Relation to the Wassermann and Kahn Tests. As the antigenic extracts used in the complement fixation test for infection with *E. histolytica* were not prepared from pure cultures of the parasite, as these are not yet available, and contained extractives not only from the amœbæ but also from the bacteria growing in the cultures and from the culture medium, it was thought possible that the positive results obtained might be caused by non-specific substances in the extracts, as is the case with the antigens used in the Wassermann and Kahn tests for syphilis, in which non-specific substances in the extracts of the tissues used are capable of reacting with substances in the patient's blood serum and fixing complement. In order to obviate this source of error, and to ascertain whether the positive results obtained might be due to syphilis, or not, a check of as many of the sera tested as possible was made by ascertaining the result of the Wassermann and Kahn tests, as well as the complement fixation test for amœbic infection, and it was found that eleven, or eighteen per cent, of the cases giving a positive complement fixation reaction for *E. hystolytica* also gave a positive Wassermann and Kahn reaction, while forty-nine, or 81.9, gave a negative Wassermann and Kahn reaction. On the other hand, of 481 cases giving a negative complement fixation test for *E. histolytica*, forty, or 8.3%, gave a positive Wassermann reaction, and forty-three, or 8.9%, gave a positive Kahn reaction, while 441, or 91.7%, gave a negative Wassermann reaction, and 438, or approximately ninety-one per cent, gave a negative Kahn reaction.

In interpreting these results it should be remembered that the vast majority of the sera tested were obtained from our serological laboratory and from patients whose blood sera had been submitted for a Wassermann and Kahn test, many of them from the venereal wards of the hospital. Thus, it is not surprising that the percentage of positive Wassermann and Kahn reactions is comparatively high in both the cases giving a positive and a negative complement fixation test for amœbic infection. However it is true that the percentage of positive results with the Wassermann and Kahn tests in the cases giving a positive complement fixation reaction for *E. histolytica* has been approximately ten per cent higher than in the cases giving a negative complement fixation test for *E. histolytica*. It is also significant that of the eleven cases giving a positive reaction with all three tests, no less than six did not show *E. histolytica* in the feces. In view of these findings it is probable that, in rare instances, cases giving a positive reaction with the Wassermann and Kahn tests will also give a positive reaction with the complement fixation test for *E. histolytica,* and in patients who give such a reaction, and in whom the parasite cannot be demonstrated, the positive result should not be considered as diagnostic. However, repeated microscopic and cultural examinations of the feces should be made for *E. histolytica* before considering the positive reaction as a false reaction.

While such cross-complement fixation apparently occurs, in rare instances, with this test, it is not surprising because of the crude nature of the antigenic extracts that are used, and it will not be until extracts can be prepared from pure cultures of *E. histolytica,* that this source of error will probably be obviated. Admitting that such false reactions may rarely occur, it is evident from the results of this series of experiments that, in the vast majority of cases, the complement fixation reactions obtained with the antigens employed were specific in nature and were not caused by non-specific substances in the antigenic extracts analogous to those producing the positive complement fixation reactions with the antigens employed in the Wassermann and Kahn tests.

Discussion of Results. The results of the experiments detailed in this contribution demonstrate that specific antibodies are produced in the blood serum of man in infections with *E. histolytica* and it is significant that the patients giving the strongest complement fixation reactions were those showing the mildest symptoms of the infection, or no demonstrable symptoms, while those having severe dysentery gave weaker reactions, thus indicating that complement fixation is an index to the resistance of the individual or the severity of his reaction to the parasite. It is also interesting to note that the positive complement fixation reaction disappears promptly after appropriate treatment and the disappearance of the parasite, thus demonstrating that if this is an immune reaction, it disappears very soon after the disappearance of the infection. In view of the fact that there is no evidence of immunity to infection with *E. histolytica*, as shown by the fact that reinfections are very common, it is evident that complement fixation in this infection cannot be considered as an index of immunity but that it is rather an evidence of the presence of living amœbæ in the tissues of the host.

The exact nature of the bodies producing complement fixation in this infection is unknown at present, but it is evident that the substances present in the antigenic extracts which are used, are similar in their nature to those present in other alcoholic antigenic extracts, which depend, so far as known, upon specific lipoids in the extracts which react with specific lipotropic substances in the blood serum of the infected individual. These substances, which are all characterized by the fact that they can be extracted with alcohol, are of much interest and have attracted a great deal of attention in recent years. As long ago as 1915, I demonstrated that alcoholic extracts of cultures of *Mycobacterium tuberculosis* fixed complement when used as antigens with the blood serum of tubercular individuals, and this observation has since been confirmed by Dienes and Scheff (1926), Lewis (1927), and Furth and Aronson (1927), who have demonstrated that there is a distinct substance in this bacterium, extractable with alcohol, which fixes complement when used as an antigen with the blood serum of tubercular patients. Similar alcohol-soluble substances have been demonstrated in *Corynebacterium diphtheriæ* and *Streptothrix* by Freud (1927); in *Treponema pallidum* by Craig and Nichols (1912); in the cercaria of *Bilharzia* by Fairley (1927); and in extracts of the intestine of rabbits filled with the trophozoites of coccidia by Chapman (1929).

In this connection the recent work of Kurt Meyer and of Sachs, Klopstock, and Wile, is of interest. Kurt Meyer has shown that antisera obtained by injecting rabbits with watery extracts of tapeworms will fix complement in the presence of alcoholic extracts of the worms, while Sachs, Klopstock and Wile have demonstrated that rabbits, when injected with alcoholic extracts of rabbit kidney and pig serum, will develop an antibody to their own tissue lipoids.

It is probable that the substance, or substances, in the alcoholic extracts of *E. histolytica* used in this series of experiments, are similar to the bodies in the alcoholic extracts mentioned above and it will be possible, when pure cultures of this parasite are obtained, to determine whether specific antibodies are formed in the blood of man in response to injections of alcoholic extracts of such cultures.

The practical value of the complement fixation test in the diagnosis of infections with *E. histolytica* is still undetermined, and is greatly limited by the difficulty of making the antigenic extracts and the small amounts obtainable. The cultivation of *E. histolytica,* while not difficult in trained hands, is not easily accomplished by the average laboratory technician, and the maintenance of such cultures for weeks and months, as is necessary in order to keep such antigenic extracts on hand, is a matter of considerable technical difficulty. In addition, not every extract made from the cultures is antigenic, for we have several times obtained inert extracts from the same strain of *E. histolytica* that had given and afterwards gave excellent antigenic extracts, although the inert and active extracts were prepared in the same manner.

Clinically, and as checked by the results of feces examinations, the test would appear to possess a high degree of specificity, and in its use in hospital practice here has repeatedly proven its value in demonstrating amœbic infections that would otherwise have remained unrecognized. The test does not give a positive reaction in other diseases or infections, except in rare cases of syphilis, as proven by the fact that such reactions have not been encountered in the hundreds of cases of miscellaneous diseases and infections that have been tested during the progress of these experiments, so that it may be stated that it is a very highly specific test when positive, and almost as specific in demonstrating the absence of *E. histolytica,* when negative. However, our experience has been that practically all individuals giving a positive reaction with the test have also showed *E. histolytica* in the feces, so that the diagnosis of the infection could have been made by a fecal examination. In many patients, however, such an examination would not have been requested, owing to the absence of, or mild character of the symptoms of amœbic infection present, or their resemblance to those of some other disease condition, and it was in such cases that the test proved of greatest value, and will prove of the greatest value when used as a routine procedure in diagnosis.

CHAPTER XXII

EXPERIMENTAL AMŒBIASIS IN KITTENS

By

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In the study of any human disease the discovery of a susceptible laboratory animal marks an important step forward because such a discovery makes possible the general application of experimental methods. Experimental work is still further facilitated when the organism known to be the etiological agent may be grown in culture. For human amœbiasis Hlava (1887) found the kitten susceptible but thirty-eight years elapsed until Boeck and Drbohlav (1925) discovered a practicable method of cultivating the pathogenic amœbæ. From fifty to seventy-five per cent of kittens between the age of weaning and the attainment of a weight of 600 to 700 grams become positive when injected rectally with fecal material or culture fluid containing the parasites. Infection of kittens also results from ingestion of cysts as in man. Also, as in man, secondary lesions develop in the liver. The kitten harbors no naturally occurring *Endamœba* so that fecal diagnosis is simplified. It would appear, therefore, that the experimental study of this important disease may proceed at a rapid rate. It will be shown in the following paragraphs, however, that progress is still retarded by problems peculiar to the life of the kitten and the introduction of the amœbæ into them.

RÉSUMÉ OF EXPERIMENTAL WORK

Early workers did not all agree that the amœbæ which were adequately described by Loesch (1875) really produced the lesions. This disagreement was due in part to the fact that intestinal bacteria, certain species of which produce dysentery, were thought to be involved in all cases. Kartulis (1890) demonstrated that the amœbæ were pathogenic to kittens and that the associated bacteria were not. Another cause of disagreement was that differentiation had not been made between pathogenic and non-pathogenic amœbæ, of which both kinds were found in human stools. Schaudinn (1903), Craig (1905), Viereck (1907) and others demonstrated that only *Endamæba histolytica* which was known to be pathogenic in man was also pathogenic in kittens. Other investigators have attempted to attenuate or increase the virulence of *E. histolytica* by continued passage through kittens (Wenyon, 1912, Dale and Dobell, 1917). Kittens have also been used in attempts to differentiate between the extremely pathogenic and less pathogenic strains of this *Endamæba* (Wagner and Thompson, 1924, Brumpt, 1925, Kessel, 1927). Sellards and Theiler (1924) demonstrated that infection of kittens by ligating the colon and injecting the parasites through its wall was practicable and that excystation occurred when this method was used. Sanders (1928) found that kittens injected with a virulent strain of *E. histolytica* showed a slight anemia, a loss in weight and a polymorphonuclear leucocytosis plus a rise in the numbers of large mononuclear cells eventuating in the death of the animal. Terminal bacteremias were occasionally met with in these injections. The writer (1929) began a series of experiments to determine how the amœbæ produced lesions. In this work it appeared that diarrhea preceded dysentery, due to the multiplication of the amœbæ within the lumen of the colon and the response of the host to a hypothetical toxic substance which was apparently excreted by the parasites. The character of fresh lesions suggested that the amœbæ were also able to invade tissues and to cause bleeding. The ulcers appeared to commence in the surface epithelial cells and are, therefore, not preceded by abscesses as would result if the amœbæ first invaded the crypts, causing them to become occluded and necrotic. It was also demonstrated that the effects of the reaction of the host on the amœbæ may be studied morphologically. The nuclei of the latter underwent pathological changes similar to those which occurred in the injured cells of the body. Though, as Kartulis demonstrated, the bacteria do not act in a primary capacity, they appeared to work with the amœbæ as soon as the initial lesions had commenced. The frequent occurrence of bacillary diarrhea and dysentery in the controls was one of a number of complicating factors. Conclusive evidence on most of the problems outlined above has not been obtained. Their investigation is being continued in various laboratories.

FACTORS THAT COMPLICATE EXPERIMENTS WITH KITTENS

The animal body is adapted to combat disease since parasitism has no doubt occurred throughout the course of evolution. Furthermore, the body responds in a typical manner to the attack of a given parasite; hence diagnosis may be made from symptoms. The ideal of the experimental method is to study the effects of transmission of a given parasite to healthy animals, *i.e.,* those that are free from other pathogenic parasites and live under conditions that are conducive to their well being. These effects should be compared with those that occur in controls. Even so, the interplay of many unknown factors makes it necessary to repeat the experiments a number of times before much dependence can be placed on them. Unlike rats, mice, and guinea-pigs, cats are not commonly reared in the laboratory. They are taken from an environment in which they were free to roam and isolated in wire cages. Furthermore, the food that they receive is seldom the same as that which they selected before capture. The above factors affect them unfavorably. This is particularly true of kittens of the age susceptible to amœbiasis. Intercurrent infections such as pneumonia and bacillary dysentery, which would otherwise be held in check, may appear. The kittens are usually purchased from the streets; some come from alleys, others from sections where living conditions are more favorable. Kittens in different parts of the world are subject to different endemic infections which makes it difficult to compare results of different laboratories. It will be shown in following paragraphs how some of these complicating factors may be evaluated, but it is obvious that careful studies of the needs and habits of kittens should be continued in order that experiments with them may be placed on a par with those in which rats, mice, guinea-pigs and other laboratory animals have been used.

SELECTION AND CARE OF KITTENS

Very few kittens are obtainable during the winter months and laboratory conditions are very unfavorable during this season. It is, therefore, advisable to plan the experiments so that a given series may be completed in early autumn. The animal room should be well lighted so that kittens may enjoy direct sunlight during part of the day. The cages should be large and roomy and two or more kittens kept together whenever practicable. The access of cockroaches should be prevented by suspending the cages from the ceiling or by some other support. Cleanliness is very important and cannot be overemphasized. On arrival in the laboratory the kittens should be inspected for fleas and other vermin. These may be removed by the application of powdered insecticides such as calcium fluoride, or by thorough washing with soap and water With the latter treatment the room should be warm and the animal quickly dried to prevent the onset of pneumonia. Infections with helminths, coccidia and other intestinal parasites may be detected by microscopic examination of fresh fecal material, and kittens that are heavily infected should not be used. The action of vermifuges such as oil of chenopodium, or carbon tetrachloride is so severe that an animal so treated is unfit for use during the age of susceptibility to amœbiasis. Fresh cows' milk if available is the most desirable food and it should be supplemented with inexpensive meats. A regular feeding schedule should be adopted and uneaten foods promptly removed. It is a good practice to weigh the kittens every morning before feeding, because loss of weight indicates indisposition which may lead to more serious illness. When the above suggestions are followed infected kittens usually live from four to six weeks instead of from three to nine days as has been so often reported.

OPERATIVE PROCEDURE

The method generally followed of inoculating the kittens is by rectal injection. A rubber catheter and a Luer syringe are required. Injury to oneself may be prevented by putting the kitten into a sack of heavy cloth which closes with a drawstring. Hard fecal pellets must not occur in the colon at the time of this injection. Some investigators give the animal a laxative on the preceding day. If this is done the dose must not be too large as diarrhea may result. A mild irrigation of the colon with a jet of warm water from a vessel supported several feet above the kitten will also effectively remove these pellets. This treatment should precede the inoculation by several hours. As a rule not more than five cubic centimeters of fluid containing amœbæ should be inoculated. In the article by Boeck and Drbohlav (1925) the reader will find a practicable method of transferring amœbæ grown in culture to the syringe. When fecal material is used the only prerequisite is that it be sufficiently fluid to pass through the catheter. Amœbæ in feces of infected kittens are more effective as inoculum than are amœbæ grown in culture. Kessel (1927) recommended that the kitten be held head downward for about fifteen minutes after treatment. Other workers have suggested the closing of the anus by a plug of cotton coated with collodion or a purse-string ligature. Both of these operations cause constant annoyance to the kitten until it finally removes the obstruction. If the injection is skillfully performed the animal may be subsequently left undisturbed. Kittens inoculated as described above usually become positive within three to five days. Loss of weight and diarrhea are the first manifestations but trophozoites of *E. histolytica* must be demonstrated in the feces to establish the diagnosis. It is much easier to detect the living amœbæ in wet preparations than to locate them in stained smears. At room temperature, 25° C., the amœbæ will live a number of hours and, if each kitten is in a separate cage, fecal samples may be collected from the latter. Under other conditions a warm enema of normal salt solution must be given to secure suitable material. The method of inoculation after laparatomy, referred to above in the review of the literature, involves a surgical operation. Skill to carry out the above can be acquired only by special training. A description will therefore not be attempted in this article.

RECORDING AND PRESERVING OF DATA

Naturally the objectives of any given series of experiments will determine the nature of the experimental data to be emphasized, but the following suggestions are of general application. The value of well preserved tissue with proper data pertaining thereto cannot be fully estimated at the time when taken. They frequently have a bearing on problems of the future. For example: the detection of the living amœbæ in a wet smear satisfies the requirements of a positive fecal diagnosis, but one may wish to know at a later date whether pathological changes were detectable in the nuclei of these amœbæ. A well-stained smear will serve to determine this point and also to confirm the accuracy of the diagnosis. Cytological details are best brought out with Heidenhain's iron-hematoxylin. Directions for preparing and using this stain will be found in another chapter. It cannot be too strongly emphasized, however, that fixation must be performed immediately while the smear is still wet. The general tendency of beginners is to make the smears too thick. Another practice that should be followed without exception is the conducting of careful autopsies. Unfortunately many students of general parasitology lack adequate training for this work. Intestinal epithelium undergoes dissolution very rapidly after death, hence intestinal tissue should be fixed immediately. If the colon is opened out in a Petri dish in normal salt solution the feces may be gently washed away and the lesions detected. A binocular microscope may be used if necessary. Excellent fixation can be secured with hot Bouin's fluid poured on after the organ has been fastened flat to a piece of cardboard. After half an hour or longer transfer is made to 70 per cent alcohol in a vial properly labeled. Sectioning may be accomplished without removing all of the picric acid. For the preservation of liver tissue a thin slice through an entire lesion and the peripheral zone is cut with a sharp knife. Avoid handling with other metal instruments. Immerse the slice immediately in a fixative, preferably hot Schaudinn's or Zenker's fluids. After twenty-four hours of hardening, the fixative must be removed as directed in guide books. Lungs, kidneys, spleen, etc., may be handled the same as above. Data are more effectively brought to the attention of other workers through the use of condensed tables and graphical representations than through the publication of detailed protocols. Furthermore, the making out of such summaries gives one a clear perspective of his own work. Training in statistical mathematics is very valuable in this field.

SUGGESTED PROBLEMS

It will be seen from the foregoing discussion that each step in advance has reopened problems that appeared settled by earlier work. For example, the successful cultivation of the amœbæ suggests that they may live within the lumen of the colon without access to living tissue. Further experimental work on this question is in order. As demonstrated by Dobell and Laidlaw (1926), Yorke and Adams (1926) and Dobell (1928) the amœbæ may complete their life cycle in the test tube. This suggests an explanation for the "carrier" condition in man but does not preclude the probability that certain naturally occurring strains are less pathogenic than others. Recent work by Dobell and Laidlaw (1926) and Rees (1928) suggests that infectivity and pathogenicity may be influenced by certain conditions of culture. This is also an interesting question. Unweaned kittens and older cats are refractory to infection but the causes of the phenomena are unknown. The general opinion of pathologists is that amœbæ do not call forth the typical inflammatory reaction of the host, but this opinion requires further investigation. Careful records of the number and relative proportions of the blood cells in amœbiasis are needed. Histological studies of secondary liver lesions in kittens will yield interesting results. The study of amœbiasis may also yield fundamental information concerning the origin and causes of pathogenicity. Very little is known about this subject in the fields of bacteriology, protozoölogy or helminthology. Why is it that many parasites live within the host without apparent injury to the latter while others produce disorders that may lead to death? An amœba unlike a bacterium has an organization that may be studied microscopically. The fruits of research in histology and cytology may be applied. Therefore there is a bright future for work in experimental amœbiasis when healthy kittens may be always available and all stages in the life cycle of the parasites are obtainable in cultures.

CHAPTER XXIII

PROBLEMS AFFECTING THE DIFFERENTIAL DIAGNOSIS OF THE DYSENTERIES

By

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INTRODUCTION

The methods of cytodiagnosis have so long been successfully employed in the diagnosis of conditions that either directly or secondarily affect the blood-vascular and hæmapoietic systems that we no longer question the broad principles upon which they rest. The same may be said of the cytological and histological criteria upon which rest our ability to distinguish between tumors of various types, and of the inferences that may be drawn from the microscopic study of spinal fluid in certain morbid conditions. The possibilities of these procedures are now established to the point that they have become very useful, while their limitations are understood to a degree that has made their application scientific.

More recently, there has developed another field for the adaptation of cytology to diagnosis. That field is found in the cytological study of intestinal discharges, under various conditions of disease. Its most successful application, so far, has lain in the differential diagnosis of the dysenteries—bacterial and protozoal, but sufficient has been learned in the past few years to show that it has far broader possibilities, and its extension to a wider range of pathological conditions is predestined.

It is difficult to say just when the significance of the cytology of bowel exudates was first suspected. I find abundant evidence of its study in Woodward's monograph (1879). Bahr (1912) laid some stress on it in his monograph on dysentery in Fiji, and allusions to it had previously been made by other authors. Rogers

(1928) also has referred to his early work with it. For the most part, however, the exudate either was ignored or erroneous interpretation of the appearances were made. A study of the papers of Thomson and Thomson (1916) and of Bartlett (1917) will show to what extent misconception and misinterpretation have gone and will save the student from similar errors, especially after he has compared the descriptions of these authors with those of Bahr and Willmore (1917-18) and Willmore and Shearman (1918). Other publications that should be consulted are those of Anderson (1921), Woodcock (1920), and Haughwout (1924*a* and *b*), and Haughwout and Callender (1925). There is not space to go into details here, but the subject is adequately covered in the papers I have named. The principle may be broadly stated in a single sentence:

The cellular exudate from the bowel is a replica of the histopathology of the condition.

Dysentery of bacterial origin is an acute inflammatory, toxic process. The bowel wall, accordingly, undergoes the reactions characteristic of acute, toxic inflammation and the exudate derived from it naturally shows the same characters. Therefore, we know we are dealing with an organism that produces inflammation and toxic necrosis.

On the other hand, we find that the mode of attack on the bowel wall by *Entamœba histolytica* and *Balantidium coli* is of an entirely different nature. Here, destruction of tissue *by the parasites* occurs only in their immediate neighborhood. It is a non-toxic proteolysis by enzymes elaborated by the parasites, and is not, in any sense, an inflammatory process. Any inflammatory reaction in such cases always is attributable to secondarily invading bacteria.

Thus are laid down the basic criteria for our inquiries into the differential diagnosis of dysenteries and diarrheas. The reactions of cellular structures to invasion by pathogenic bacteria are sharply expressed. The reactions of identical structures to the attacks of pathogenic protozoa are strikingly different and, in the cases of *Entamœba histolytica* and *Balantidium coli* our knowledge is sufficient to give us a basis for comparison.

This would seem to open the way for easy sailing, but, unfortunately, the course is far from clear. Much remains to be learned, and much confronts us that must be disproved and then unlearned.

This refers not so much to the recent progress in cytodiagnosis as to the rich store of primeval lore that has found its place in medical practice and even in supposedly serious scientific literature.

There are, to be sure, many perfectly legitimate unsolved problems bearing on the diagnosis of dysentery that hang on incompletely elaborated fact; but there also are, alas, many others conceived in ignorance and born in misapprehension that, none the less, now require to be dealt with in serious and scientific fashion in order that we may arrive at a semblance of the truth concerning them. Had I the space I gladly would review the brilliant achievements of clinical medicine, pathology, bacteriology and medical zoölogy in the field of intestinal pathology. They have given us a solid footing on which we shall be able to deal with a great mass of superficial, misdirected and inaccurate effort, that has embellished the literature during the past twenty-odd years. The product of this effort has been the formulation of a congeries of amazing speculations and fantastic problems which, in certain quarters, pass current for the last word. However, I must devote this space to an exposition of what I consider to be the more important unsolved problems in the microscopic diagnosis of the dysenteries. Many of these questions have been raised largely by the method, or lack of method, employed by certain investigators. While a number of them have no real merit, all must sooner or later be disposed of by scientific method. This is as much for the correction of the moral effect they have produced as anything else.

Before we can hope to settle these questions we must travel far afield and carry our quest far beyond the mere consideration of animals in their rôle as the real or fancied producers of disease. The picture of dysentery is a panorama not a vignette. No one can study amœbic dysentery and close the problem at its borders, missing all acquaintance with bacillary dysentery. Neither may one study the group of reactions legitimately comprehended under the term dysentery and ignore the other numerous acute and chronic affections of the intestine that cloud the issue. Accordingly, if I seem to stray from the narrow path indicated by the title of this chapter, and if I seem extravagant in my views as to what factors must be weighed in dealing with the broad aspects of our problems, I can only ask that the reader suspend judgment until he has compared supposition with the fruits of a wider observation and the analysis of concomitants.

To begin, let me state my belief that the evidence in our possession to-day points to *Entamœba histolytica, Balantidium coli* and *Eberthella* (*Bacillus*) *dysenteriæ* as being the only organisms which have been proved to cause dysentery in the strict sense. There are, to be sure, numerous other PROTISTA, and a few METAZOA, that have been named as causes of dysentery or, at least, said to be related to the phenomena of dysentery, and they furnish the greater number of the unsolved problems relating to the diagnosis of dysentery. Many of the remaining problems are furnished by a group of so-called constitutional diseases of uncertain etiology, part of the symptomatology of which is referable to the alimentary tract. Anemia, of the primary type, may be mentioned as one of the more important members of the latter group.

Adequate inquiry into the phenomena and causes of these conditions, therefore, involves excursions into the fields of zoölogy, bacteriology, pathology, serology and clinical medicine. Accordingly, diagnosis of the dysenteries may be said to rest upon three factors: the microörganisms present; the evidences of local reaction in the intestine as manifested in the bowel discharges, and the general physical state of the patient as ascertained by clinical study *which should include the consideration of all concomitants.* In other words, in the study of a given organism to determine if it is the cause of dysentery, it not only is prudent—it is important—first to ascertain with certainty whether a state of dysentery exists. Moreover, the mere presence of a pathogenic organism in the bowel contents of a person suffering from an intestinal disorder does not necessarily settle the question of diagnosis—on the contrary, it may complicate it exceedingly. The phenomena must be studied from a broad viewpoint. Not only must the organisms present be studied, the reactions of the infected person must be interpreted and an effort made to harmonize the two.

BACILLARY, AMŒBIC AND BALANTIDIAL DYSENTERIES

In laying down our problems let us start with bacillary dysentery for it affords the most striking and easily analyzed picture of all the acute intestinal conditions.

Preëminent among the problems to be solved is the determination of criteria that shall make it clear to the clinician when the administration of serum is essential or, in advanced cases, when it is likely to be of avail. I need not go into the old controversy over the limitations of serum therapy. It is sufficient to say that the prevailing opinion is that serum is not effective if given later than three to five days after the onset of the attack. I think this is true in a large proportion of instances, but I also am growing to believe that many cases are benefited by serum given even later. Moreover, I think a more detailed knowledge of the finer characters of the bowel exudate in these late cases, and also in the very early cases, is likely to give us something to work on.

All this requires a more definite knowledge of the significance of the varying proportions that the different cells in the dysentery exudate bear to each other. In a frank bacillary dysentery, the polymorphonuclear neutrophiles usually greatly outnumber the other types of cells present. Yet, there are frequent instances in which epithelial and endothelial cells dominate the picture and the neutrophiles, although numerous, furnish a much lower proportion. I have not been able to draw any definite conclusions regarding this. Bahr (1918) regards the persistence of the endothelial macrophages in the exudate as the case progresses as indicating active process of repair. Occasionally, though, one encounters a case that continuously produces a high proportion of macrophages, and at the same time runs a long and severe course. Study of this point is of real importance.

Mononuclear cells of a distinctive type—not lymphocytes—frequently are seen in bowel exudates. By some they are spoken of as plasma cells, by others as "irritation cells," although their relation to the so-called Turck's cell is not made clear, and hematologists tend to regard Turck's cell and the plasma cell as representing two different types. For myself, I am in doubt as to what they are. I do know, however, that in certain instances, such as I have described (1924*b*), they dominate the picture. I am convinced they are of great importance in certain conditions.

Eosinophile leucocytes often make their appearance in considerable numbers, both in amœbic and in bacillary dysentery. At times it is to be suspected that their presence is associated with serum reactions or with helminthiasis. In other instances, there seems no explanation for their presence in such large numbers. Their relation to intestinal allergy, or atopy, will be discussed elsewhere.

The main factors concerned in the diagnosis of uncomplicated amœbic dysentery are now generally understood. The old chronic cases, however, occasionally present features that make it a matter of difficulty to distinguish between an old amœbic process with a heavy secondary bacterial infection of amœbic ulcers, and a late stage of bacillary dysentery. As a matter of fact, both are secondary manifestations of a similar nature and for purposes of treatment the uncertainty is not of great moment. It must be remembered that unchecked amœbic processes may lead to marked anatomical changes involving the entire structure of the bowel wall. With the secondary bacterial invasion that always accompanies this, come cellular manifestations, sometimes slight—sometimes marked. In the marked instances there are extensive necrotic cellular changes (karyorrhexis, karyolysis, etc.), and one even may find an occasional macrophage. Better criteria are needed for the interpretation of these pictures.

Furthermore, an effort should be made finally to define the significance of Charcot-Leyden crystals. Acton (1918) has discussed them and his conclusions, in which I concur, were conservative. More recently, Thomson and Robertson (1921*a* and *b*) have taken the extreme view that their presence is pathognomonic of *Entamœba histolytica* infection. To my mind, the question is still open. The remarks of Connal (1922) concerning the finding of these crystals in a case of infection with *Isospora hominis* are of interest. As a matter of fact, the appearance of Charcot-Leyden crystals in the feces is quite as irregular as that of cysts of *E. histolytica* and they lack the positive value that attaches to finding the latter.

Balantidial dysentery is of comparatively rare occurrence. Accordingly, the studies of the bowel exudate in that condition that have been published by me (1920 and 1924*a* and *b*) are based on very few cases. For that reason, no opportunity should be lost, by the careful study of other cases, to check my findings. The situation is complicated by the designation of two species of *Balantidium* in the pig by McDonald (1922). It should be ascertained if both *Balantidium suis* and *B. coli* will parasitize man and, if so, if both are capable of producing dysentery. Settlement of these questions may explain numerous conflicting views as to the pathogenicity of *Balantidium* to man. The reader is referred to the papers of Strong (1904), Walker (1913*b*), and Manlove (1917).

Acton and Knowles (1924) and others lay some stress on the chemical reaction and the pH findings in dysenteric stools. Anderson also (1921) has called attention to the clumping of erythrocytes in the exudate of acute amœbic dysentery. Further study of these things may develop points of value in diagnosis as well as treatment and prognosis. Jacoby (1920) has discussed the question of the reaction of bacillary exudates and as his findings differ somewhat from those of Acton and Knowles, his paper should be consulted.

Two questions now arise: First, are intestinal protozoa other than those I have named, capable of producing dysentery? Secondly, do they make their attack upon the tissues in a manner different from that of *Entamœba histolytica* and *Balantidium coli* and is the tissue reaction different from that seen in amœbic and balantidial dysentery?

To illustrate: Invasion of the intestinal wall of the ground mole by *Cyclospora karyolytica* is stated by several authors to produce a severe, even fatal, "enteritis." Has the nature of this infection been sufficiently studied to justify us in citing *Cyclospora* as an example of a protozoön that *produces* inflammation? What part do bacteria play in the case? May not the same reservations be made concerning *Eimeria zurni*, which is supposed by some observers to *produce* a similar condition in cattle.[1] The case of *Trichomonas* I shall discuss later.

That brings us to still another question: If these animal parasites (other than *E. histolytica* and *B. coli*) do not produce dysentery in the strict sense of the term, are they capable of initiating other pathological conditions that simulate dysentery to an extent that involves them in differential diagnosis? Much so-called evidence has been advanced in support of the view that many or all of these organisms, particularly the flagellates, are pathogenic. Most of this literature is of an exceedingly unscientific and unconvincing nature. Some of it I have reviewed (1918).

It would appear, then, that in attacking the general problem of the differential diagnosis and classification of the many and varied intestinal disturbances in man, we may proceed under the following assumptions which form a sufficiently established scientific basis of attack:

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[1] This is apart from the action of secondarily infecting bacteria.

Disturbances accompanied by marked evidence of reaction to inflammatory and toxic processes are of bacterial origin.

Disturbances manifested by evidence of proteolytic destruction of tissues, hemorrhage and mild simple inflammatory reaction of a secondary nature, are caused primarily by animal organisms.

Our unsolved problems, real and fancied, fall into a group of disturbances that is not sharply marked off in this manner. They have been stated by numerous writers, sometimes fairly conservatively, but often with gross extravagance. Out of a large number of such papers that I have read in the past few years I have selected two which illustrate what I mean. I refer to the papers of Smithies (1926) and of Barrow (1924).

Host-parasite relationships have been discussed in this volume and elsewhere by Hegner, and I have touched on the matter of the chemistry, evolution and general theory of these relations in other publications (1914, 1918, 1920). Much of this matter is rather speculative, but it will be well for the student to go over it in formulating his problem.

THE INTESTINAL FLAGELLATES AND THE QUESTION OF THEIR PATHOGENICITY

There is evidence that under certain conditions, so far not ascertained, some trichomonads will wander from their natural habitat in the intestine to other parts of the body of their host. This makes the case of this flagellate the most interesting of our borderline problems. Haughwout and de Leon (1919) have reported finding *Trichomonas* and spirochetes in the exudate aspirated from the pleural cavity of a Chinese in Manila. However, careful study of sections of the lung and pleura failed to disclose any in these situations. The origin of the flagellates in this case is extremely uncertain. The case reported by Kessel (1925) is more convincing. He found *Trichomonas* in the exudate from the liver of a Korean at operation for hepatic amœbiasis. It seems not unlikely that they accompanied the amœbæ from the intestine to the liver through the portal circulation. Wenyon's discovery (1920) of *Trichomonas* in the mucosa of the intestine at autopsy furnishes some evidence that this flagellate is capable of invading the tissues of man. It is singular, however, that other similar findings have not been made. It already has been shown by Haughwout and de Leon (1919), Kofoid and Swezy (1924), and others, that *Pentatrichom-*

onas will ingest and digest erythrocytes. In the discussion that has arisen over the interpretation of this, there has been a tendency on the part of some writers to miss the point which, to my mind, is of great importance—*that the trichomonads digest the corpuscles.* The general run of trichomonads are vegetarians and the fact that certain of them can digest and assimilate animal food I have interpreted (1920) as possibly indicating a trend of evolution towards obligatory tissue parasitism. It is quite true, as Hegner (1928) and others say, that the *mere ingestion* of erythrocytes is not proof of pathogenicity. In all cases where I have observed it, the symptoms clearly were caused by agents other than the flagellates. The liver abscess in Kessel's case undoubtedly was of amœbic origin.

While I believe that the case against the intestinal flagellates including those of the trichomonad group has been grossly overstated by many writers, still, more definite evidence must be developed before we are justified in ignoring them entirely as possibly contributing to confusion in the diagnosis of dysentery. For myself, I can only say that I have seen many hundreds of heavy trichomonad infections and never yet have found reason for suspecting them as a cause of dysentery. In the instances in which I have observed them to ingest erythrocytes, the corpuscles, as I have stated, clearly have been the accompaniment of a typical dysentery.

Many writers credit *Giardia* with pathogenicity. Disturbances attributed to the activities of this organism range all the way from frank dysentery to simple diarrhea. It has been suggested that the end-products of its metabolism are absorbed by the intestinal villi and produce undesirable symptoms. Other suggestions are that its close application to the intestinal epithelium may retard normal absorption or provoke diarrhea as a response to irritation. Definite evidence of any of these assertions remains to be developed. If any of these conditions can be shown to be caused by *Giardia,* methods for the differential diagnosis of them apart from other intestinal disturbances will have to be devised.

There is even less evidence against *Chilomastix, Enteromonas* and *Embadomonas.* Being less widely known among medical practitioners they have enjoyed comparative immunity from implication in obscure intestinal conditions. *Entamœba coli, Endolimax nana, Iodamœba bütschlii* and *Dientamœba fragilis* are so dearly commensal in their mode of life and their morphological characters are so well defined that I do not consider they afford any problem in diagnosis to the competent worker.

The questions that may arise over the pathogenicity of *Councilmania lafleuri, Karyamœbina falcata, Entamœba paradysenteria, E. macrohyalina, Caudamœba sinensis* and *Craigia* may conveniently be deferred until settlement of their systematic position. Their identity as valid species rests under doubt.

THE COCCIDIA, PLASMODIUM, LEISHMANIA AND THE INTESTINAL SPIROCHETES AND FUNGI

Although we have little if any direct evidence concerning coccidial infections in man, our general knowledge of the COCCIDIA assumes that the epithelial cells of the small intestine are invaded by *Isospora hominis.* The only problem that concerns diagnosis lies in the determination as to whether a condition in any way resembling dysentery develops during the course of an infection with *Isospora,* or whether the abnormal bowel discharges that have been noted by several observers are attributable to other causes. It is conceivable that a massive infection might strip the mucosa bare of epithelium and thus open the way for an acute bacterial invasion. However, I know of no instance of this in man.

Several instances of infection with *Isospora* have now been studied. At least four observers, Noc (1920), Haughwout (1921), Connal (1922) and Schule (1927), have noted a periodic diarrhea recurring at rather regular intervals which may be associated with periods of schizogony in the epithelial cells. The occurrence of epithelial cells in the stools of persons infected with *Isospora* also has been reported, and in one or two instances coccidia were said to have been seen in some of the cells. However, it is as yet unproved that either the periodic diarrhea or the desquamation of the cells were caused by the coccidia. In fact, Connal, in the account of his case, raised grave doubts as to the origin of cells in that manner. Moreover, it is questionable if intracellular coccidia would be recognizable after their long journey from the site of infection in the small intestine to the anus.

Most of the earlier and some of the more recent writers on tropical medicine recognize dysenteries of malarial and leishmanial nature. I never have had the opportunity to study a suspected case of leishmanial dysentery, but I have been able to investigate a few that were thought to be malarial. I can say with confidence that I consider every one of these "malarial dysenteries" to have been a perfectly typical bacillary dysentery. Moreover, I believe it is the opinion of the greater number of experienced men in the tropics that all so-called leishmanial and malarial "dysenteries" can, on a little careful study, be resolved into dysenteries of amœbic or bacillary origin.

At the same time, definite proof that *Plasmodium* and *Leishmania donovani* are incapable of giving rise to dysentery has never, so far as I am aware, been adduced. It is well known that these parasites may be found in the blood vessels of the intestine, and many practitioners believe that they may give rise to dysenteric symptoms. That being the case, we might expect to find a bowel exudate of the amœbic type. Careful search should discover the parasite in the exudate, and trophozoites of *Entamœba histolytica* or *Balantidium coli* should be absent.

Dysentery occurring in subjects known to be infected with *Leishmania donovani* or *Plasmodium* should be studied with especial care until sufficient evidence has accumulated to settle the question beyond reasonable doubt. It must be borne in mind that diarrhea, and gastric and intestinal hemorrhage may occur in malaria under circumstances that make it likely they are directly associated with the disease. These usually should cause no confusion. Moreover, a simple hemorrhage from the bowel is not dysentery. It is the bloody, mucoid, frankly dysenteric exudates that are most likely to lead to errors in diagnosis. The application of drugs usually employed in malaria and leishmaniosis cannot be expected to be effective against either protozoal or bacillary dysentery. It will thus be seen that the settlement of these questions is of real importance.

Spirochetes are frequently found in feces. They have been reported as occurring in a variety of conditions ranging from cholera and dysentery to the so-called post-dysenteric colitides. Several species have been described, but I am not convinced that all of them are valid. There is need of further study of these organisms in their possible relation to dysentery. Students should consult the papers of Thomson and Thomson (1914), Crowell and Haughwout (1918), Taylor (1919) and Hogue (1922), Crowell and Haughwout briefly review the literature from 1880 to 1916.

A few words must be said of the yeasts and fungi of the intestinal tract. These organisms have been reviewed as a group by Anderson (1917). His paper includes a large bibliography. He discusses *Parasaccharomyces ashfordi* which many regard as the cause or at least an important contributing cause in psilosis or sprue. No student of intestinal disorders should neglect to acquaint himself with some of the literature on sprue. Ashford's paper on the etiology of the disease (1917) should be read as an introduction to the subject. The papers of Christian (1925), Newham (1926), and of Reed and Ash (1927), are of equal importance.

Because of its superficial resemblance to the cysts of amœbæ, *Blastocystis* is a source of confusion and error to the inexperienced. Lynch (1922) recognizes two and possibly three species and in a later paper (1923) suggests that it may be pathogenic. Other writers have voiced the same opinion, but there really is little, if any, evidence in support of this. It is of almost universal occurrence in the stools of persons living in the tropics and is very common elsewhere. As in the case with the flagellates it appears in increased numbers in loose and diarrheic stools. This coincidence is worth pondering upon.

With the possible exception of the sprue organism there appears to be no evidence implicating any of these forms in intestinal pathology. However, *Blastocystis* is still suspected by some of being pathogenic and its position in this respect should be finally determined.

It is a matter of real importance, however, to define the cellular picture seen on the rupture of the intestinal abscesses in schistosomiasis (bilharziasis). The problem is similar to that presented by intestinal involvement in malaria and kala-azar. Several writers have mentioned the general character of this exudate, but the best account I have so far seen is that of Faust and Meleney (1924). Bahr (1918) brings out a few points that should be borne in mind. The discovery of the ova of the parasite will, of course, define the primary condition. The problem is to establish the general character of the exudate so it may be distinguished from that of an intercurrent amœbic or bacillary dysentery which might proceed to a fatal termination under bilharzia treatment.

EXUDATES IN MALIGNANT, CHRONIC AND CONVALESCENT CONDITIONS, CHOLERA, INTESTINAL ALLERGY AND THOSE RESULTING FROM CHEMICAL AND MECHANICAL IRRITATION

Much remains to be done in the study of cellular exudates in malignant, chronic and convalescent conditions. I already have described some of these pictures (1924*a* and *b*), but more study is needed. The papers of Thorlakson (1924) and of Bargan (1924) should be consulted for views concerning primary ulcerative colitis. Low (1920) has written a short paper in which he describes a number of confusing conditions which have been encountered by many microscopists who have had occasion to do diagnostic work in dysentery. These include the appearances presented by syphilis, tuberculosis, malignant disease and various surgical conditions. Occasionally, also, one encounters strange reactions in the intestinal tract produced by powerful poisons, such as mercury and arsenic. I have described a case (1922) of violent intestinal irritation produced by the ingestion of uncooked *Colocasia esculentum,* a common foodstuff of the tropics.[2]

Allergic reactions in the intestinal tract produced by certain proteins ingested by susceptible persons are described by Duke (1922). They are frequently encountered and are a fruitful source of error in microscopic diagnosis. I have called attention to the type of bowel exudate that occurs in these cases (1924*b*). It is characterized by the presence of eosinophile leucocytes and plasma or "irritation cells" which make up the bulk of the cellular mass. This is in harmony with the nature of the disturbance and permits a differential diagnosis from dysentery. More work should be done on it, however. Zahorsky (1926) has laid stress on the importance of this type of exudate in pediatrics.

Another line of comparative investigation lies in the thorough study of the bowel discharges in cholera, through all stages of the disease. Maitra, Ganguli and Basu (1925) have written a short paper on their findings in India, but their microscopical methods were faulty and more careful work is needed. Goodpasture (1923) has given a good account of the histopathology of cholera and, as his descriptions are based on the study of tissues fixed and stained by approved methods, they form a good foundation for the study of the appearances seen in the bowel discharges.

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[2] The fine hairs from the sheath of the bamboo *Dendrocalamus strictus* Nees, have been administered in food with homicidal intent. They produce a suppurative condition suggesting a chronic colitis. Powdered glass gives rise to similar effects.

SUGGESTIONS FOR THE STUDY OF UNSOLVED PROBLEMS IN DIAGNOSIS

The foregoing comprises a brief review of what the writer considers to be the outstanding problems in the laboratory diagnosis of dysentery. With settlement of the etiology of amœbic, balantidial and bacillary dysenteries, attention is becoming focused on the more obscure aspects of the general problem, which consequently begin to emerge as essential features of the broader picture. We shall now proceed to an equally brief consideration of viewpoint and method involved in their possible solution. Considering only the work that has led us astray and raised false problems, it would seem that in the past too much solitary, non-correlated work has been undertaken. Moreover, there has been lack of attention to many of the less obtrusive details which were submerged during the developmental period of the work on dysentery. Accordingly, the investigator who wishes to inquire into any of the conditions we have been discussing will, if he would make his study effective, establish an entente with capable (and, I might add, interested) clinicians. He also will seek the cooperation of men in pathology and bacteriology on whose knowledge and interest he can rely.

The selection of clinical material upon which to base a series of studies of any of these problems should be made with the most scrupulous care and thoroughness. Failure to do this may undermine the whole project. If the problem at hand seems to have many ramifications, it will be well to be persuaded in the beginning that all of them must be thoroughly explored. Of course, it may take years to do this which, unfortunately, is not in harmony with the prevailing ambition to achieve quick results and early publication.

The first essential is a thorough and searching physical examination and a careful inquiry into the medical history of the subjects. Particular attention must be paid to the anal and rectal regions and the abdominal wall should be carefully palpated with a view to discovering regions of tenderness and other abnormalities. In work of this kind it often is necessary to make inquiries of other institutions and former medical attendants. However, data secured from such sources should be critically reviewed. This work should not be delegated to a young hospital interne or a clinical clerk. It should be done by the clinical man of the group, and such work as he may require to be done by others should be carefully checked by him. This care and thoroughness in the clinical observations should be continued throughout the studies.

If the laboratory work on the subjects is well done, it is highly probable that it will be discovered by this time that a considerable number of candidates will be unsuitable for further study, for no matter how promising their crop of parasites and their intestinal symptoms may appear at the outset, many inevitably will find their place in the group of diseases in which the basic pathology is clearly out of the field under investigation, or in which it is generally conceded that intestinal symptoms are secondary to a disease of extra-intestinal nature. This process of elimination is one of the most important and, I may add, one of the most frequently neglected features in studies on these problems.

Apart from the study of the bowel discharges, which I shall discuss separately, I should regard the following as about the minimum amount of laboratory investigation each subject should receive:

The Blood. The study of the blood should include careful total erythrocyte, leucocyte and differential counts, the study of well-stained films, hemoglobin estimation, and the color index. It is not an indifferent matter how this is done. The literature bears evidence of the lack of harmony there has been in the methods employed in the study of the blood. Much of the work on blood counts and hemoglobin estimations in connection with the maladies we have under discussion has been done by such varying and haphazard methods as to leave us in a position of extreme uncertainty as to the facts. This is shown in the paper of Fischer (1919) who made a study of the blood in amebic dysentery, and that of Lampe (1923) who made similar observations in bacillary dysentery.

In order that comparison may be made with the pictures shown in the primary anemias and sprue, blood studies are needed in the diarrheas of various types (including "flagellate diarrheas"). The counts should be made during non-diarrheal periods as well as during exacerbations. This should yield information regarding the extent of the elevation of the erythrocyte count by diarrhea—a factor that often is overlooked.

It is, perhaps, unnecessary for me to dwell upon the unreliability of the prevailing methods of hemoglobin estimation. The factors of error are as great as those affecting blood counts. The Sahli, Dare and Tallqvist methods are, perhaps, the most commonly used. None is entirely satisfactory. The principle of the Dare instrument is good, but I have found inaccuracies in the depth of the pipette, an account of which I have published (1929). Ideally, we should use some such method as that of Van Slyke (1918, 1921), but it is too involved for use in a long series of cases unless special study of the blood is being made. Probably the best method for work of this kind is that of Newcomer. If the color disc is correctly standardized this is an extremely accurate method. The proportion of hemoglobin present always should be recorded in grams per 100 c.c. of blood—not by percentage. Whatever method of hemoglobin estimation is selected should be used throughout the work. The study of the blood in such problems could scarcely be considered to be complete without resort to blood chemistry in appropriate cases. The significance of ionic calcium deficiency in sprue is at present very uncertain, and it is my belief that further investigation is desirable.

All work on the blood should be done by a person of wide experience in hematology—not a student, nurse or technician. I would urge, also, that all blood estimations be made with apparatus of known accuracy.

The Urine. It should receive a thorough general examination. All determinations should be made on a 24-hour specimen. The usual general examination should include qualitative tests for indican and urobilinogen. It is recommended that the student consult the papers of Rowntree, Hurwitz and Bloomfield (1913) and of Fleming (1926). Further work on the urine will be guided by the nature of the case.

In special cases it may be desirable to determine the basal metabolism of the subject, especially after long residence in the tropics. Fleming's observations (1925) will be found suggestive here. The clinical man will employ the sigmoidoscope and the Roentgen rays as occasion arises. It would be inappropriate to do more than mention these measures here.

Investigations of this kind naturally focus around the intestinal findings. Accordingly, work on the feces and bowel exudates should be done with the greatest thoroughness and accuracy. Needless to say, such work should only be undertaken by a person of broad knowledge and experience. The bowel contents should be studied daily during the course of the investigation.

There is not space here to go into the technique of coprology. Consequently I shall only emphasize a few points, and earnestly urge the careful study of the methods set forth in Cammidge's admirable book (1914). The recent literature on biochemistry, nutrition and related subjects also should be consulted. Incidentally, I might point out there is great need for revision and systematization of the terminology of coprology.

Collecting the Specimen. I know of nothing more important in such work than the proper collection of bowel evacuations. The microscopist should be satisfied with nothing less than the entire movement. A portion of the movement selected at random by a nurse or by the patient himself, far from yielding adequate information may even be misleading. Some stools consist of separate formed and unformed portions. If the microscopist receives a sample of the formed portion he records the stool as normal and knows nothing of the pathological elements that may be in the other portion. If he receives the unformed portion he records the stool as diarrheal and possibly misses other equally interesting things. The stool should not be allowed to stand after its passage, but should immediately be sent to the microscopist.

Preferably, the movement should be obtained without the aid of artificial means. All are objectionable. Castor and mineral oils offer great obstacles to microscopic work. The drastic purgatives produce watery stools that complicate the picture in many ways and add greatly to the difficulty of fixation and staining of the material on the slide or coverglass. Watery movements very rapidly bring about changes in delicate organisms that render them unfit for study. Enemata are quite as bad. When there is difficulty in getting a specimen, a glycerine suppository sometimes will stimulate the lower bowel to activity. A full dose of strychnine sulphate often is effective. When these things fail, one may give fluid extract of cascara in very moderate dosage, or a merely laxative dose of Carlsbad water. The latter I prefer to anything else, but it must not be given in sufficient quantity to produce a watery movement. The point is to obtain a movement with the least alteration of its prevailing characters. These matters naturally will be looked after by the clinical man.

The so-called "provocative methods" employed in the detection of infections with *Entamœba histolytica* require little attention. If they possess any virtues at all these lie in the field of clinical medicine. For humane reasons some of them never should be used. I mention them only that I may emphasize the point that such methods have no place in research.

Careful macroscopic examination of the specimen should be made *as soon as it reaches the laboratory* and careful note should be made of the general physical characters.

We shall now briefly consider a few points of importance in the study of bowel discharges.

To begin: It is vitally important to distinguish between *stools* and *exudates*. A "stool" must contain fecal matter. The mucoid masses passed in dysentery are *not* "stools." However, an exudate may be found in a stool, or, the entire movement may consist of exudate. Exudates are the product of inflammation and are composed of fluids and cellular elements derived from the tissues of the subject. Fecal matter does not enter into their composition. These distinctions should be accurately recorded.

There seems also to be some misapprehension as to the use of the term "acholic." It often is bestowed on pale stools which, on application of a chemical test for urobilin, are shown to contain an abundance of that substance. It will be seen, therefore, that the eye does not furnish a reliable means of determining whether or not a stool is acholic. Accuracy in this respect may be very important; therefore, it is wise to apply Schmidt's mercuric chloride test before pronouncing a stool acholic. More exact work will require the application of the Wilber and Addis method.[3]

Dysenteric movements frequently are atypical. In the strict sense they are small and mucoid and often blood-streaked, but that is usually when the attack is well under way and the bowel has been cleared of fecal matter. Amœbic dysentery movements often are feculent, loose and blood-streaked. On the other hand, the evacuations of bacillary dysentery may be watery or serous, and the truth may not be apparent until the mucus has been carefully examined microscopically.[4] Late in the course of a fulminating dysentery, when the epithelium has been stripped from the bowel, the movements will contain no mucus and little else besides blood and serum.

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[3] The stools of sprue often are spoken of as acholic because of their pale coloration. As a matter of fact, the characteristic color of sprue stools is due largely to their high content of fat. Moreover, the bile pigments become converted to "colorless urobilinogen." This substance always occurs in excess when a severe aplastic anæmia complicates the sprue.

Movements of bacillary dysentery, especially those of the tenacious mucoid type, frequently contain so little blood that it is discovered only on microscopic examination. This is misleading to the uninitiated and frequently leads to the assumption that the condition is not one of dysentery.

Purulent movements are encountered in suppurative conditions in the intestine following dysentery or consequent upon a variety of surgical or malignant conditions. Much work remains to be done on stools of this type because of the confusing pictures they present in connection with the diagnosis of dysentery. The base of these stools usually is feculent, but the accompanying exudate is purulent in the true sense. Frequently the pus is found closely adherent to the fecal mass, in which event it usually is mixed with mucus of a tough, tenacious kind, or it may occur as a separate portion consisting of a creamy mass of leucocytes and other cells. It is quite similar to the drainage from a suppurating wound.[5] I already have described types of these movements in the publications cited.

Needless to say, no study of these conditions can be complete without tests for occult blood. The benzidin reaction is simple and sufficiently sensitive for preliminary work. It should be applied with the subject on a diet free from meats and green vegetables. The sample of stool under examination should be carefully extracted with *neutral* ether before the test is applied in order to get rid of the fats.

We now come to a flagrantly neglected, though vitally important, phase of this work. I refer to the study of *post-mortem* material and, I may add, it should not be too far *post mortem.* Wenyon's brief study (1920) is the only recent instance I can recall where real use of such material has been made in connection with the study of the flagellates. Material of this kind could afford a valuable means of control on these studies. Undoubtedly, much of it can be obtained by an alert man who keeps track of the patients in a charity hospital, many of whom are available for necropsy immediately after death.

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[4] No matter what type of stool is under examination, search always should be made for mucus and if it is found it should undergo careful microscopic examination. The preparations always should be stained.

[5] A mistake that one frequently comes across in the literature is the use of the word purulent when the more correct term would be foul. Purulent signifies inflammation and pus, whereas foul may signify nothing worse than a bad smell. The error may be most misleading for a purulent stool is the expression of definite pathology, while foul may be applied to a variety of stools that are not pathological in any sense.

It is not generally appreciated what excellent material may be obtained in the state prisons and penitentiaries. Once official barriers are passed there usually is no difficulty in getting the men to volunteer for experimental work. A certain proportion of them always will be found to be infected with intestinal parasites, and as all of them are under good sanitary control, the opportunities are excellent for well controlled work, such as Walker (1913*a*) performed at Bilibid Prison in Manila. Particularly valuable are the opportunities afforded by men condemned to death.

It is here that there is great promise of clearing up obscure points on the relation of intestinal parasites to the host tissues and where we may find our explanations of reactions—or the lack of reactions.

In doing this work I have found it desirable to organize the performance carefully and rehearse it until everything runs smoothly and quickly, for when the formalities are over and the cadaver has been released by the authorities, the work must be done quickly if the best results are to be obtained.

The first requisite is to secure the aid of a pathologist who is expert at *post-mortem* technique. He should clearly understand the nature of the study that is to be made that he may plan his work so that the microscopists and others shall receive their material quickly and in good order. There should be a sufficient number of persons present to carry out the operations without delay. The exact sequence and methods to be used will have to be governed by the nature of the case.

I consider it unnecessary to describe the technique that will be employed in the study of living and fixed organisms and tissues. Dr. Andrews has written on cytological and other technique in another chapter of this book, and men undertaking such problems naturally will be trained in the work. Moreover, methods will have to be evolved as obstacles are encountered. Such things cannot be anticipated here. The literature is very rich on technical methods and an attempt to adequately summarize it here is not to be considered. However, the study of the monographs of Dobell (1917, 1919), and of Walker (1913*a*), is strongly recommended.

This closes a rather diffuse discussion of some of the unsolved problems affecting the diagnosis of the dysenteries. I have gone into many things that may appear to have very little to do with the subject. That is because it is my belief, that our first task under existing circumstances, lies in the studied elimination, by thorough methods, of a number of imperfectly studied factors and vaguely conceived assumptions that have been accepted as partially, if not wholly, true simply because they have been put forth so many times. They have made the problem as a whole seem more vast than it probably is. To dispose of these fallacies, further knowledge must be gained on maladies that bear no relations whatever to dysentery. To many, this will seem a very cumbersome and indirect way of going about it. To me, it seems the only way left to us. After all, our real object is the lessening of suffering and the saving of life, which in this workaday world must even displace the transcendent joy of arriving at abstract truth; and yet, wherein lies the difference? I am not thinking of the highly trained physician who is in close touch with other specialists and works by modern methods; I am thinking of the submerged ninety per cent who have been systematically schooled in error these many years, and who have been led to lean heavily on generalities because they were not in the way of getting anything else.

It is my belief that if this sketchy program is carried out, not only will considerable light be thrown on the validity of certain unknown factors affecting our methods of diagnosis of dysentery, but in the process a wealth of other information will be made available for other studies of equal importance. No effort or detail will be wasted if we can arrive at that happy end. Truly enough, it is difficult to deal with such broad and elusive problems, and to those young investigators (and perhaps a few of the older ones), who may feel themselves oppressed by the task, I would commend a careful reading of that most attractive and inspiring text of scientific method—deKruif's *Microbe Hunters* (1926).

CHAPTER XXIV

PARASITIC INFUSORIA IN GENERAL

By

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Although most of the infusoria are free-living in habit a number of parasitic species occur in each of the larger groups. Problems and methods of study with respect to the genus *Balantidium* and the opalinidæ will be found in the two following chapters. Most of the protozoa that occur in the stomach of ruminants are ciliates; these are described in Chapter VI. Information regarding certain species of ciliates is also presented in several other chapters; the reader will find references to these in the index. Recent accounts of the morphology and classification of the infusoria are presented in Wenyon's *Protozoology* (1926, pp. 1153-1229) and in Calkins' *Biology of the Protozoa* (1926, pp. 363-401). Calkins has also included a key to the genera of infusoria (pp. 401-414). Wenyon's account deals largely with parasitic forms whereas Calkins includes both free-living and parasitic species.

Authorities differ with respect to the classification of the infusoria. As a rule the group is divided into two sections, the ciliata and the suctoria. Wenyon follows Metcalf in dividing the group into the protociliata, containing the single family opalinidæ which are all parasitic, and the euciliata containing the ciliata and suctoria. Four orders are usually recognized in the section ciliata. The following paragraphs are intended to indicate in brief to what groups the parasitic ciliates available for study belong and in what types of animals one may expect to find them.

The family opalinidæ belongs either in the suborder astomina of the order HOLOTRICHIDA or in the group PROTOCILIATA, depending on opinion. All of the members of this family are parasitic. The best account of the group is contained in Metcalf's (1923) monograph on the *Opalinid Ciliate Infusorians.* Problems and methods of study of this family are presented by Metcalf in Chapter XXVI. These ciliates, as Metcalf shows, are very easily obtained from the rectum of frogs and other AMPHIBIA and offer many interesting and important problems for study.

The order HOLOTRICHIDA contains many species of parasitic ciliates. All of the species belonging to the suborder ASTOMATEA are parasitic in habit, living principally in the intestine and body cavity of invertebrates. A number of species have been described from the intestine of earthworms. Some of these are known by the writer to occur in the earthworms of Baltimore. A species belonging to the genus *Intoshellina* has been reported from the freshwater worm, *Tubifex,* and a species of *Haptophrya, H. michiganensis,* has recently been described from the intestine of a newt in Michigan (Woodhead, 1928). A species belonging to the genus *Cepedella* occurs in the liver of cyclad molluscs of the genus *Sphærium.* Considerable information is available regarding *Collinia branchiarum* which is parasitic in the body cavity of *Gammarus pulex.* Whether this and other species that have been recorded from the body cavity and intestine of various invertebrates occur in this country is not known.

The suborder STOMATEA contains a large number of interesting species. Several of these belong to genera such as *Chilodon, Colpoda* and *Uronema* that have been reported from human feces and have been considered by some as human parasites. These should, however, be classed as coprozoic species. The interesting ciliate *Ichthyophthirius multifiliis* that lives in the skin of fishes belongs in this suborder. Many species of STOMATEA have been reported from the cecum of the horse, from the cecum of wild guinea-pigs and from the rumen of cattle and sheep.

To the order HETEROTRICHIDA belong two genera of great interest. These are *Nyctotherus* and *Balantidium.* The genus *Balantidium* is considered in Chapter XXV. Several species of *Nyctotherus* have been described from man but these, if they really belonged to this genus, were probably coprozoic forms. Species of *Nyctotherus* from animals are easy to secure. *N. cordiformis* is present in the rectum of frogs, a large percentage of which are infected. How many species of this genus occur in frogs it is difficult to state. A careful study of the genus should be made. Another species, *N. ovalis,* occurs frequently in the common cockroach and in the mole cricket.

To the order OLIGOTRICHIDA belong a number of ciliates that occur in the stomach of cattle, in the cecum of the horse, in the cecum of wild guinea-pigs, in the intestine of the chimpanzee and gorilla and in the intestine or cecum of certain other mammals. In this order belong the common genus *Diplodinium* which was described in detail by Sharp (1914) and *Troglodytella* which was first described by Brumpt and Joyeux (1912) from the chimpanzee. The latter is one of the few protozoa of monkeys that has not been found in man (Hegner, 1928).

The order HYPOTRICHIDA contains comparatively few parasites. These usually creep about on the surface of aquatic invertebrates. One of the best known species is *Kerona pediculus* which Uhlemeyer (1922) has shown to be an obligate parasite.

There are also comparatively few parasites among the PERITRICHIDA. A number of species of the VORTICELLIDÆ attach themselves to aquatic animals and may be considered parasitic in the broad sense of the term. Others, such as *Spirochona,* occur on the gills of the fresh-water crustacean *Gammarus*. The species most easily obtained for study is *Trichodina pediculus* which creeps about on the body of fresh-water hydra or tadpoles, and other aquatic animals. Another ectoparasitic peritrich is *Cyclochæta* which lives on the skin of fish.

Most of the SUCTORIA attach themselves to aquatic plants, to animals, particularly small crustacea, and insect larvæ. All degrees of parasitism are exhibited by members of this group. Certain species attach themselves to almost any object, either living or lifeless; others commonly are found attached to plants; some prefer snails, others protozoa, water insects, hydroids or crustacea; in some cases any part of the body of the host is apparently satisfactory but frequently only one particular region is selected. Certain members of the group actually parasitize other protozoa. The SUCTORIA are thus seen to vary greatly in their host-parasite relations.

CHAPTER XXV

THE GENUS *BALANTIDIUM*

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INTRODUCTION

SPECIES of the genus *Balantidium* have been reported from man, the chimpanzee, orang, baboon and other Old World and New World monkeys, domesticated animals (pigs, cattle, sheep, horses, guinea-pigs), a bird, a tortoise, salamanders and frogs, fish, crustacea (sand fleas), insects (cockroaches and *Culicoides*), snails, annelids, turbellarians and cœlenterates. They are particularly abundant in pigs, guinea-pigs and frogs. Many species have been described from these various animals. Specific differences are principally shape and size of the body, shape and position of the cytostome, and shape and size of the macronucleus. *Balantidium* is rare in man except in certain localities (*e.g.,* Aguilar, 1925, in Guatemala) and is not frequent in monkeys, but is common in pigs and guinea-pigs. The species from man, monkeys and pigs have for many years been considered to belong to one species (*Balantidium coli*), and recent studies (Scott, 1927) indicate that the species in the guinea-pig is also *B. coli*. McDonald (1922) believes that a second species, *B. suis*, occurs in pigs, but it seems probable that he was dealing with exconjugants which have a larger ratio of length to breadth than specimens during growth stages.

SOURCES OF SUPPLY

Balantidia are common in pigs. McDonald (1922) reports an incidence of infection of sixty-eight per cent among 200 pigs in California and Nevada, and at least as high an incidence occurs among pigs in Baltimore. Among guinea-pigs the incidence of infection depends on the source from which the animals are secured; sometimes almost all of a number of specimens may be infected and at other times none or only a few. Clean guinea-pigs can be infected by feeding them cysts. The trophozoites of balantidia from pigs will live in the cecum of rats for at least several weeks when injected by laparotomy (Schumaker, 1929) and will persist for at least several days in the ceca of young chicks when injected *per rectum* (Hegner, 1929). Balantidia from both pigs and guinea-pigs can be maintained in artificial media in test tubes. Many species of balantidia have been reported from frogs and Wenyon (1926) states that frogs are "nearly always infected," but little success has been had in obtaining specimens from these animals in the United States. Only in certain tropical regions can balantidia be obtained readily from man or from monkeys. Cysts seem to be rare in man, rare in pigs, at least at certain times of the year, not so rare in monkeys, but common in guinea-pigs.

OBTAINING AND PREPARING MATERIAL FOR STUDY

a. Obtaining material. Pig. *B. coli* can be obtained from pigs in slaughter houses at almost any time. A glass fruit jar or vacuum bottle, piece of cheesecloth, and microscope, either a compound microscope with low power objective, or better, a binocular microscope, should be available. The vacuum bottle may be rinsed out with water at body temperature (37° C.) before use so as to prevent any cooling of the intestinal material put into it. The ciliates are most abundant in the cecum and upper three feet of the colon. The cecum is a sort of dilated pouch into which the small intestine opens. In the cecum there may be as many as 120,000 balantidia per cubic centimeter; fewer trophozoites usually occur in the colon, which is about six to eight feet long. Some trophozoites escape in the feces probably because the fecal material is often soft and moist. Cysts may be looked for in the lower part of the colon but are difficult to find, if present, because of the large number of worm eggs and débris in the contents. The cecal or intestinal material should be strained through cheesecloth into the container to remove larger particles; it is then ready to be transported to the laboratory. There is no evidence that lesions are produced in the intestinal wall of the pig hence the procuring of tissue is not necessary.

Guinea-pig. Balantidia may also be obtained from guinea-pigs, in which they occur in the cecum and upper colon in the trophozoite stage and in these locations and in the lower colon in the cyst stage. Cysts appear to be common in the more solid fecal pellets. The balantidia in the cecum of the guinea-pig seem shorter and broader than those in the pig, but this is probably due to the nature of the environment, since they resemble the latter when both types are grown in the same culture medium.

Monkeys, man, etc. In certain localities balantidia may be obtained from monkeys and man (especially in certain tropical countries) or from frogs or insects, but pigs and guinea-pigs are the best sources of supply.

b. Viability under laboratory conditions. That trophozoites of *B. coli* are very resistant to cooling is indicated by the work of Rees (1927) who noted that at room temperature (about 20° C. = 68° F.) the organisms remain active and apparently normal for from four to eleven days; other workers (McDonald, 1922; Jameson, 1927) state that these ciliates die within a few hours when brought into the laboratory. It is probably always best to use fresh material for study or else to grow the ciliates in culture media. Specimens are more numerous at the bottom of the fecal mass, probably because they are anaërobic or react positively to gravity.

c. Cultivation. B. coli from man was first cultivated by Barret and Yarbrough (1921) and later by van der Reis (1923). The medium of Barret and Yarbrough consisted of one part of inactivated human blood serum to sixteen parts of 0.5% salt solution. About 0.1 of a cubic centimeter of undiluted feces was placed at the bottom of tubes ten millimeters in diameter and 150 mm. in length containing about eight cubic centimeters of the medium; they were then incubated at 37° C. Maximum growth took place in from forty-eight to seventy-two hours and subcultures were inoculated every second day over a period of thirty-two days, when the cultures were allowed to die out. Cysts were present in some of the cultures; binary fission was common; and what appeared to be conjugation was observed on one occasion.

A similar medium to that described above was used by Rees (1927) in his work with *B. coli* from the pig. A modification of a Ringer's solution (NaCl 6.5 gms., KCl 0.14 gm., $CaCl_2$ 0.12 gm., $NaHCO_3$ 0.20 gm., and Na_2HPO_4 0.01 gm., per liter of distilled water) was substituted for the 0.5% salt solution and human or horse serum or Lœffler's dehydrated blood serum (eight grams to 100 c.c. of distilled water) added; pig serum proved unsatisfactory. Cultures were maintained for over fifty-four days at 36° C., transplants being made usually at three-day intervals. Trophozoites were sometimes present in a culture at the end of seven days but their number usually decreased rapidly after three days. The addition of a minute quantity of sterile rice starch was found to be favorable for the growth of the balantidia and was ingested by them with avidity. A pure line was obtained by picking out single specimens with a micropipette, placing them in a drop of culture medium on a coverslip and dropping this into culture tubes.

B. coli from the pig was also cultivated by Jameson (1927). He found the Boeck and Drbohlav (1925) medium for the cultivation of *Endamœba histolytica,* as modified by Dobell and Laidlaw (1926), to be most satisfactory. This consists of inspissated horse serum with Ringer's solution, normal saline (0.9%), or Ringer's solution with the addition of egg albumen as liquid constituent. The addition of egg albumen is particularly favorable for starting a culture but plain Ringer's solution is superior for maintaining the culture. Difficulty was experienced in starting cultures without the presence of sterile rice starch; this substance, according to Jameson, inhibits the growth of *Blastocystis,* a common intestinal fungus, which is detrimental to the balantidia. The hydrogen-ion concentration of the culture medium is important; at a pH of 5.0 or below, the organisms die and a pH of over 8.0 is unfavorable. Unfavorable bacteria may be eliminated from cultures by the addition of a few drops of a 1 in 10,000 solution of acriflavine, and dirty cultures clear up if argyrol is added so as to produce a strength of 1 in 2000.

Balantidia from the guinea-pig have been cultivated by Rees (1927) and Scott (1927). Rees found this to be more difficult than with balantidia from pigs, but finally succeeded with the same medium. Guinea-pig balantidia are more active in cultures than in the cecal material taken directly from the animal. They ingest starch readily and resemble the balantidia from the pig in appearance. Cysts were noted in tubes from which trophozoites had disappeared, indicating that encystment occurred in the cultures. Scott used Locke's, Ringer's or 0.75% NaCl solutions for cultivating balantidia from the guinea-pig. A small piece of intestinal wall in a one-half strength Ringer's solution maintained the ciliates and allowed them to multiply by fission. Room temperature (20° C.) was satisfactory and a low oxygen supply and reduced light were found to be favorable. By means of transfers to fresh tubes the balantidia were kept in cultures for from three weeks to one month.

d. Preparation for study. Living trophozoites and cysts. A sample from the bottom of the intestinal material should be placed on a slide, slightly diluted with normal saline solution, and confined under a coverslip. The following characteristics may be determined by a study of the living trophozoites: size, shape, length and arrangement of cilia, ectoplasm, endoplasm, peristome, cytostome, cytopharynx, cytopyge, contractile vacuoles, food vacuoles (?), nuclei (?), methods of locomotion. The cysts are nearly spherical and within the cyst wall the slowly moving cilia can be seen.

Stained trophozoites and cysts. Heidenhain's iron-hematoxylin is the most satisfactory stain following fixation in Schaudinn's solution. Eosin, orange G and licht grün may be used as counter stains. Alum cochineal is also satisfactory for general study and Mallory's triple connective tissue stain is good for nuclear differentiation. The surface of the balantidia can be stained by Bresslau's method (Scott, *1927*). A very small drop of a ten per cent solution of China blue is placed on a slide and mixed with a drop of culture medium containing the ciliates. This is allowed to dry slowly at room temperature. During this period particles of the stain are ingested and stain the food vacuoles and ciliary furrows. When dry the preparation is cleared in Xylol and may be mounted in gum dammar or kept uncovered in a dry state. Sections may be made of washed balantidia embedded in paraffin or of the intestinal contents containing balantidia in celloidin. Sections should also be made of invaded tissues when these are available.

PROBLEMS FOR INVESTIGATION

1. *Morphology and Life-Cycle.* a. Structure. Certain purely zoölogical problems that have come to the attention of the writer are presented here under the heading of Morphology and Life-Cycle. The most detailed account of the structure of any species of *Balantidium* is that of McDonald (*1922*) of *B. coli* from the

pig. This investigator describes both ectoplasmic and endoplasmic structures and especially the neuromotor apparatus. Similar studies are needed of balantidial cysts, and of balantidia from other animals. Certain chromosome-like bodies that are constant in number and size were discovered by Hegner and Holmes (1923) in the macronuclei of balantidia from a Brazilian monkey; this work suggests that a further cytological study of the nuclei of these ciliates would be of value.

b. Size. Balantidia from different hosts and from a single host vary greatly in size; for example, *B. coli* from man ranges from 30μ to 200μ or more in length and from 20μ to 70μ in breadth. Are these variations due to fission and subsequent growth, to the nature of the environment, or are heritably diverse size races or varieties present? Pure line cultures and cross-infection experiments would probably answer this question. Rees (1927) was able to obtain a pure line culture of the *Balantidium* of the pig in one of twelve attempts in the following way: a large drop of culture medium containing a few organisms was spread out on a slide and placed on the stage of a binocular dissecting microscope. Then a single balantidium was sucked up into a fine pipette, and forced out on to a piece of coverglass. This was quickly examined, before it had a chance to dry, so as to be certain that only a single specimen was present, and then the coverglass, with the balantidium on it, was dropped into a culture tube. Rees reports multiplication, but did not succeed in making a transfer to a second tube. The pipette may be of the ordinary type with a rubber bulb on one end and the other end drawn out in a flame until the diameter is much reduced, or two short pieces of glass tubing may be used, connected by a rubber tube about 6 cm. long, the free end of one tube being sealed and that of the other tube being drawn out fine. This sort of pipette enables one to regulate the suction better than does the type with a bulb at one end.

c. Specificity. The specific validity of the many balantidia that have been described is somewhat in doubt. A careful study of the entire genus *Balantidium* is desirable from this viewpoint. The structure of specimens from the various hosts needs to be worked out as well as the life-cycles and host-parasite specificity. Confirmation is needed of McDonald's conclusion that two species of *Balantidium* live in the pig, *B. coli* which belongs to the same species as that in man, and *B. suis,* which is a new species.

Measurements of conjugants, exconjugants, and recently divided specimens should be made to aid in determining this point. Pure line cultures started with *coli*-like and *suis*-like specimens respectively might settle the question. Confirmation is also needed of Scott's assertion that the balantidia of the guinea-pig are specifically identical with *B. coli* of man.

d. Fission. Binary fission has been observed frequently in material taken directly from animals as well as in specimens grown in culture media, but the details of the process have been described only in a general way (Scott, 1927) and in a limited number of species.

e. Conjugation. Scott (1927) and Jameson (1927) have recently published data on conjugation in balantidia from the guinea-pig and pig respectively, but much work still needs to be done with these types as well as with balantidia from other animals. Conjugation took place in Jameson's cultures periodically, the intervals observed being in some cases three days and in others one week, but the conjugants did not encyst as described by Brumpt (1909). Do conjugation cysts actually occur? What factors induce conjugation? Is conjugation usually periodic?

f. Budding and Sporulation. Does budding occur, as described by several investigators (for example, Ohi, 1924) and sporulation (Walker, 1913)?

2. *Host-Parasite Relations.* a. Transmission. Data on the viability of trophozoites of *B. coli* when outside of the host are contradictory (McDonald, Rees). A series of experiments might well be undertaken to determine the factors involved, and their effects on trophozoites and cysts. It is generally supposed that man becomes infected by swallowing cysts from pigs; the truth of this supposition should be determined experimentally. Are trophozoites infective? Hegner (*1926*) found that trophozoites from the pig would pass through the stomach and small intestine (120 cm.) of the guinea-pig and reach the cecum in a viable condition within one hour; this was confirmed by Rees. It is still to be learned whether or not trophozoites are able to reach the colon of larger animals, such as the pig and man, alive. The conditions that exist within the digestive tract might be simulated in the laboratory and the ciliates subjected to them, especially in cultures. The effects of treating balantidia both in pigs' feces and in culture with hydrogen sulphide have been studied by Rees but with variable results.

b. Excystation. No one knows in what part of the digestive tract excystation occurs, how long a time is required to bring it about, nor what factors are responsible for the process. An easy method to study excystation is to inject cysts into the stomach of laboratory animals through a rubber catheter attached to a syringe and then examine the stomach and intestinal contents at intervals of about an hour. Work *in vitro* might also succeed, as it has in the case of excystation in certain amœbæ and flagellates.

c. Pathology and Pathogenesis. The pathology of the lesions due to *B. coli* in man is fairly well known but the origin of the lesions (pathogenesis) is still in doubt. Walker (1913) states that balantidia, when they come in contact with a solid object, do not back off and try another direction, as *Paramœcium* does, but attempt to bore their way through; he suggests from this that balantidia bore their way mechanically into the tissues of the intestinal wall. Another possible method of tissue invasion is that of dissolution of the cells by the secretion of cytolytic enzymes. If a laboratory animal can be found that can be infected with balantidia and in which these ciliates are pathogenic, this problem can be studied effectively by setting up infections and observing the formation of the lesions in the early stages. Neither guinea-pigs nor pigs seem to be injured by balantidia although Brumpt (1909) obtained lesions in a young pig which he infected with balantidia from monkeys. Scott finds that balantidia invade the tissues of guinea-pigs after the death of the host, but this is of little value in studying pathogenesis in living tissue. Perhaps young pigs or monkeys might be used for studies on pathogenesis.

The effects of balantidia on other body tissues and upon the physiological processes of the host are little known. Laboratory animals, for example, monkeys, that are known to be susceptible, could be infected and the chemical constitution of the blood and changes in the cellular content of the blood studied.

d. Carriers. The carrier condition exists in infections with *B. coli* as in most other protozoan diseases. Do the balantidia live as harmless commensals in the lumen of the gut during the carrier period, or do they attack the tissues, but not severely enough to induce symptoms? What constitutes the resistance of the host during the carrier period and what changes are effective in breaking down this resistance? These are primarily physiological problems involving both the host and the parasite. The results obtained by

Rees and Jameson with balantidia in cultures containing starch suggest a method of studying the physiology of the parasite. The carrier condition in the host would have to be investigated in some animal, such as the monkey, that is susceptible and sometimes exhibits symptoms.

e. Encystation. Cysts are said to be rare in man and monkeys but common in the pig. Rees (1927) reports cysts as rare in pigs in Baltimore during February and March. Perhaps the number of cysts formed varies with the seasons. Culture methods afford an opportunity to determine factors that are responsible for encystation.

f. Host-Parasite Specificity. Various problems of host-parasite specificity may be studied with the aid of *B. coli.* What species of hosts can be infected by the various species of balantidia and under what conditions? What relation has the age of the host to susceptibility or resistance? How can the apparent infectivity of *B. coli* to man, monkeys and guinea-pigs be explained? Are there two species, varieties or races of *Balantidium* in the pig (*B. coli* and *B. suis*)? Does the diet of the host have an influence on susceptibility? Is the size of the infective dose important?

CHAPTER XXVI

RESEARCH PROBLEMS IN THE OPALINIDÆ

By

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CILIATA
- PROCILIATA
  - OPALINIDÆ
    - PROTOOPALININÆ, binucleated
      - *Protoopalina* (nine subgeneric groups, about thirty-six described species)
      - *Zelleriella* (about thirty-eight described species)
    - OPALININÆ, multinucleated
      - *Cepedea* (six subgeneric groups, about thirty described species)
      - *Opalina* (two subgeneric groups, about thirty-eight described species)
- EUCILIATA

THE OPALINIDÆ are not only of interest in themselves, but are of peculiar interest because of the light they throw upon the origin of the EUCILIATA, whose remarkable nuclear condition (one transient nucleus, large, metabolic in function; and usually one permanent nucleus, small, carrying the genes functional in inheritance) is unique among living things. In structure the OPALINIDÆ are intermediate between FLAGELLATA and EUCILIATA.

I. MORPHOLOGY

1) The pellicle and its ridges have not been adequately studied, discrepant accounts having been given.

2) "Alveolar structure" was first described by Bütschli from *Opalina,* but this foam was not truly alveolar as described. Students

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[1] For a description of the structure and life-history of an opalinid, *Protoopalina intestinalis,* see Metcalf, 1923, introduction. For a bibliography of the OPALINIDÆ and a brief, critical review of the literature see Metcalf, 1909 (literature previous to 1908) and 1923 (subsequent literature through 1922).

of the structure of protoplasm might well include both ectosarc and endosarc of opalinids.

3) The inclusions in the cytoplasm need much further study. *a)* In the ectosarc are one, two, or three sorts of these bodies. Are these divergent conditions of the same thing, or are there two or three distinct sorts? Are the large blotches, sometimes giving a yellowish tinge to the living animal when seen in *white* light, especially reflected light, paraglycogen (*Cf.*, Metcalf, 1909), or what are they? Are the "ectosarc spherules" integrally united to the neuro-muscular complex (*Cf.*, Kofoid's as yet unpublished paper)? Is there any morphological or genetic relation between the spherules of the ectosarc and those of the endosarc? *b)* What are the endosarc spherules—chemical composition, function, origin? Do they arise from the chromatin of the nucleus (Metcalf, 1909) or by division of themselves (Tönniges and others)? What is the meaning of their partial or complete (?) disappearance in the gametes? How do they become restored in the growing zygote? *The whole subject of protoplasmic inclusions in the* CILIATA, *indeed in* PROTOZOA *and, for that matter, in* PROTISTA, *greatly needs study, and in any such study the opalinids deserve special attention.*

4) The "silver-line structures" (neuro-motor complex) should be studied by silver nitrate impregnation and exposure to light and also by the methods of Kofoid and his school, this especially in binucleated opalinids, in which the relations are much less complicated than in the multinucleated forms upon which Kofoid worked (Kofoid's results were described at the meetings of the Society of Zoologists, in December, 1928, but the paper is not yet published). Incidentally the results should be scrutinized to see if they give indication of the former position of a mouth.

5) The nucleus: *a)* Chromatin, metabolic and genetic; the destruction of the former at the sexual period; especially its reappearance in the growing zygote (not observed by Metcalf) and the source from which it arises and all the attendant circumstances; the greater care exercised over the division and distribution of the vegetative chromatin in binucleated opalinids than in multinucleated genera, and the interpretation of this difference; the circumstances of the ready appearance of abnormalities in the metabolic chromatin bodies ("macrochromosomes") when the animals are placed under unaccustomed conditions; the influence of different environmental factors (oxygen, pH, different foods, etc.)

upon the appearance of abnormalities. The sensitiveness of the macrochromatin to unaccustomed conditions would serve as an important index if opalinids could be reared successfully outside the host. The study of these abnormalities, the circumstances of their appearing and a comparison with the diverse appearances and behavior of the macrochromatin during the normal life-cycle, may help to interpret the normal changes. The individuality of the macrochromosomes of binucleated species only (described by Metcalf) may well be studied by another observer, since such a condition would naturally seem in itself improbable. *b*) The genetic chromosomes, strings of beads, somewhat as in *Paramecium,* need minute study. Is their number always the same as that of the macrochromosomes in binucleated species (as Metcalf found to be the case in two species of *Protoopalina* and one species of *Zelleriella,* not demonstrated in multinucleated genera)? The lengths of the genetic chromosomes and the numbers of their contained granules differ between the chromosomes and, at least in some species, the numbers of granules in different chromosomes differ by a measure greater than the margin of error in the counts in favorable material. Are these differences constant in different nuclei of the same species, and can individual genetic chromosomes thus be distinguished and followed, as Metcalf claimed was true for the macrochromosomes in the vegetative phases of the life-history in two species of *Protoopalina?*

6) Mitosis. Splitting of chromosomes, both macro- and micro-? At what stage of the mitotic cycle does splitting occur, if at all? Are the phenomena consistent with any Mendelian division and distribution of genes?

7) The plasmosome nucleolus, its nature and its relation to the cell physiology? Does it have the peculiar behavior in cell-division described by Metcalf (1909)? From what source does it arise in the second of the two daughter nuclei after cell-division?

8) The centrosome (wholly absent according to Metcalf); can its homolog be identified?

9) The mitotic spindle; is it composed of chromatin threads, or achromatin, or are there both chromatic and achromatic threads (*Cf.,* Metcalf, 1909)?

The fact that something of nuclear structure, including the chromosomes, some of the mitotic spindle fibers and the course of mitotic division, can be made out in the living animal suggests more extensive studies of these features in the living opalinid, though one must remember that the abnormal conditions under which the animal is living while under microscopic observation will soon cause distortion of at least the chromatin structures. The length of time the chromatin remains "alive" under these conditions can be tested by certain intravitam stains which do not color the chromatin until its death (*Cf. Metcalf,* 1909).

II. LIFE-HISTORY

This, while known in outline, needs restudy as to numerous important features, especially before, during and after fertilization. The behavior of the metabolic chromatin at these periods needs review, also especially the behavior of the genetic chromosomes, for Metcalf did not properly distinguish the two in his 1909 paper. This study should, by all means, be made upon binucleated rather than multinucleated forms, because of their larger nuclei, their smaller number of chromosomes and their greater emphasis, during the vegetative phases of the life-cycle, upon the macrochromosomes and their equal division and distribution to the daughter nuclei. Of especial interest are the following questions:

1) What is the origin of the chromatin spheres, one to four in number, which appear in the nucleus and are extruded into the cytoplasm during the sexual phases of the life-cycle, there to be absorbed? Are they composed of metabolic chromatin only? Is there left any residuum from the macrochromosomes after the formation and elimination of the spheres?

2) Is there a period of several generations when, as Metcalf (1909) described, there is a haploid number of macrochromosomes, or were the macrochromosomes (metabolic) and the microchromosomes (genetic) confused? What is the condition of the genetic chromosomes during this period? This needs careful scrutiny before the real nature of these important phenomena can be determined.

3) How and from what sources are the macrochromosomes re-formed in the growing zygote? There are no observations upon this important point.

4) What is the exact behavior in detail of the genetic chromosomes during the whole sexual phase of the life history? Do they split longitudinally at this time or at any other time? Are the observable phenomena consistent with Mendelian inheritance

through genes arranged in linear aggregates to form chromosomes? If not, are there genetic chromatin particles which divide, though not while aggregated in linear chromosomes?

5) If Kofoid's as yet unpublished studies of the neuro-motor complex in *Opalina obtrigonoidea* are correct, and if there are homologous structures in the binucleated genera, what is the behavior of each element in this complex during the period of emphasis upon sexual phenomena?

6) If, as Metcalf described, the ectosarc and endosarc spherules greatly diminish in number, or wholly disappear, by the time the gametes are fully formed, from what source and in what way is each kind of these cytoplasmic inclusions redeveloped? What light, if any, does the behavior of these bodies throughout the life-cycle cast on the problem of their nature and function? Does their presence during the phases of vegetative emphasis and their great diminution or complete absence during the phases of sexual reproductive emphasis indicate their special association with nutrition? Are all of the two, three or four sorts of these bodies alike in their relations to these phases of the life-cycle?

7) The growth of the zygote to the adult should be followed for each genus and for most or all subgenera, and such study should include the numerous changes of internal structure and the remarkable changes of external form, especially the series of changes by which each (?) species starts at the bottom, as a *Protoopalina* of primitive type, and climbs its own ancestral tree to reach its adult generic and specific character (*Cf.*, Metcalf, 1926).

III. HOST SPECIFICITY (*Cf.*, also the section PHYSIOLOGY)

Metcalf's review of the host-parasite relations among the OPALINIDÆ showed a general, though not sharply exact, relation between parasite genus or subgenus and host family, or in a few cases host genus. The outlines of these relations are somewhat vague, for there has been spreading of parasite groups to other than their apparently original host groups, especially in the case of certain unusually hospitable hosts, such as *Bufo*. Metcalf (1909) showed that many, and probably all, species of tadpoles in Europe can be infected by the cysts of many, probably of all, species of European opalinids, and that the tadpoles remain for months infected by apparently healthy parasites. Yet in the adult ANURA the opalinids are restricted to a limited and characteristic host distribution. This suggests a series of infection experiments, including cross-infections, with a view to ascertaining the influences which affect host-parasite comity. In such studies two features, especially, should be in mind: 1) The influences which induce rapid, repeated division and encystment of the opalinids just before the egg-laying period of the host (these processes are, of course, essential to the persistence of the opalinids from year to year); 2) the relation of the metamorphosis of the host to the welfare of the parasite.

As to the first point, one should remember that, in the tadpoles, cysts continue forming for weeks and that the same is true in the intestine of *Box boops*, the Mediterranean fish which harbors *Protoopalina saturnalis*. As to the second point, it is interesting to note that, at least for some anuran species, such as *Rana catesbeiana* and *R. clamitans*, the adults are uninfected, though the tadpoles are practically always richly infected. It should further be noted that the percentage of infected individuals among adult ANURA, of some species at least, is less, for many species much less, than among their tadpoles. *Hyla versicolor* is a conspicuous example. A review of the several species of ANURA as to the relative incidence of opalinids in larvæ and in adults might well accompany any study of the effects of metamorphosis of the host upon the infection. The change of diet in the ANURA from larva to adult and the changes in the gut during metamorphosis are, perhaps, important in this problem.

IV. PHYSIOLOGY

1) It would be of especial value if methods of culturing opalinids might be found. The animals under unfavorable conditions show distortions of the macrochromatin bodies in the nucleus ("macrochromosomes"), and they show these distortions so quickly, that these should serve as a sensitive index to the favorable or unfavorable influence of conditions of many sorts in the culture medium. The injurious effect of exposure to oxygen makes the problem of culturing a difficult one and would complicate the experimental treatment if successful culture methods were devised. Anaërobic intestinal parasites have been successfully cultured and much further study is sure to be given to the methods of such culture. The opalinids should be included in these studies.

2) Can opalinids in the host be diminished or destroyed by introducing into the intestine oxygen or substances which will give

off free oxygen (*Cf.*, Cleveland, 1925)? Try introducing by mouth, by anus, and with both adult and tadpole hosts. Can the parasites be thus destroyed without injury to the hosts?

3) The effects of different foods for the hosts upon their opalinids? Use both adult ANURA and tadpoles. Try protein diet on each, also carbohydrate diet (*Cf.*, Hegner, 1922, 1924). Do the vegetarian tadpoles give different results from the carnivorous adults? Under conditions in which protein diet favors the opalinids must fat be included in the food (*Cf.*, Stephanson's experience in the Arctic)? If so does the kind of fat present make a difference?

4) Study the effects of different sorts of bacteria in the intestine upon the opalinids (*Cf.*, Hegner, 1922, 1924; Cleveland, 1928). This is involved in food experiments. Of course the opalinids do not ingest bacteria or any other solid food.

5) Effects of drugs administered to the hosts?

6) Digestion. Do opalinids extrude digestive fluid into the cecum of their host, which acts in this blind pouch as an extracellular digestant, as Konsuloff (1922) claimed on wholly insufficient evidence, if any?

7) Excretion. The morphology of the excretory organs might well receive more extensive study (*Cf.*, Metcalf, 1907, and Konsuloff, 1922). Of especial interest is Metcalf's description of differentially staining granules associated with the posterior vesicle of the excretory system, and their actual extrusion when this contracts. This deserves further study. Is the process normal or abnormal? Is this system of organs truly excretory? Under what environmental and physiological conditions does the system increase in extent and under what conditions is it diminished? Experiment upon some of these problems is difficult without cultured animals.

8) Investigate the stimuli which induce rapid division of the opalinids and their encystment previous to the egg-laying period of the adult host (*Cf.*, the section Host-specificity). How do these conditions as to opalinids in adult ANURA compare with those affecting opalinids in tadpoles, which, of course, form no mature sexual products, but whose parasites encyst and pass to the water (*Rana catesbeiana*, *R. clamitans* and other species)? *a)* Is there in these tadpoles a special season of rapid division and encystment of the opalinids, or is the cyst-forming of the parasites more generally distributed through the whole tadpole stage of the life history of

these ANURA whose adults are uninfected? *b)* Remove gonads from male ANURA and observe effects, if any, upon the behavior of the opalinids. *c)* Do the same with the gonads of female ANURA. *d)* Treat both sexes in a similar way, only cut, or tie off, the *vasa efferentia* and the oviducts. *e)* If the opalinids show different behavior in hosts whose genital ducts have been ligated or cut from that which they show in hosts whose gonads have been wholly removed, follow the study further by introducing into such unsexed hosts gonads from other species which normally carry different species of opalinids from those occurring in the operated hosts. Make reciprocal crosses in this way. Such experiments may give some light upon, first, the nature of the stimulus which induces opalinids to encyst at their presexual period, and whether these stimuli are internal or environmental, and, second, upon the control over host-parasite specificity in infection.

9) In thyroidectomized tadpoles of ANURA which carry opalinids of a different appearance (stage of development; *Cf.*, Metcalf, 1926) in the adults and in the tadpoles, observe if in these tadpoles with delayed metamorphosis the opalinids complete their usual development.

V. SOURCES OF SUPPLY AND METHODS

For most studies the binucleated species are much to be preferred. The body is less crowded with nuclei; the nuclei are larger, their chromosomes are fewer in number, if there be any connection between a particular nucleus and any of the cytoplasmic inclusions (*Cf.*, Kofoid's paper as yet unpublished) these relations should be more readily determined; indeed the whole internal structure is less crowded and easier to study.

Material of the binucleated genera, *Protoopalina* and *Zelleriella,* is not available for study in the northeastern part of the United States, nor is it abundant in the southeastern region. In the west and southwest several species of *Protoopalina* occur, and *Zelleriella* can be had in the southwest. The author of this paper, with the coöperation of a number of zoölogists in different parts of the country, is making attempts to introduce and naturalize the European Fire-Toad (*Bombina bombina*, commonly called *Bombinator igneus*) which harbors *Protoopalina intestinalis* and *P. caudata,* species especially favorable for study. Within a year the probable success or failure of these attempts should be known, and report will be made in *Science*. Fire-Toads can be imported from Europe successfully, by parcel post or express, in tin containers only slightly ventilated. Pack about a dozen or twenty animals in wet sphagnum in each quart tin. If the toads are imported late in March or early in April they will arrive before their breeding season in May. To obtain eggs keep the toads in aquaria holding not less than a cubic foot of water, with water weeds. Collect opalinid cysts for infection of the tadpoles both from the sediment in the aquaria and directly from the recta of the toads, of course after the eggs have been laid. The toads while in captivity may be fed, on the ground, with meal worms, flour-moth larvæ, bot-fly larvæ, squirming pieces of earthworm (avoid *Allolobophora fœtida* because of reported danger of heavy coccidiosis; for the same reason avoid the portions of the earthworms which contain the sperm ripening pouches), or bits of meat.

Metcalf (1923) describes the known species of OPALINIDÆ, naming their hosts and the geographical occurrence of the hosts and the parasites, so far as known.

Opalinid material is easy to manipulate in cytological studies, responding favorably to all the usual methods of treatment. Good material of these parasites can be collected from well-preserved museum or other specimens of ANURA, alcohol specimens being much better than those in formalin. The hosts may be opened on the ventral surface without real injury and the parasites removed, with a very narrow section-lifter, from the rudimentary cæcal pouch at the upper end of the rectum (see Metcalf, 1923).

The opalinid parasites of many ANURA are as yet wholly unknown. Metcalf (1923) mentions all species which had been searched for opalinids up to that time, and but few have been explored since. Anuran tadpoles show shapes and conditions of their opalinids very different from those seen in the parasites of the adults. The life histories of the opalinids in the tadpoles are peculiarly interesting (*Cf.*, Metcalf, 1926) and should be studied for each genus and most, at least, of the subgenera. Genetic relationships between hosts are commonly indicated by their parasites. Studies of parasites are therefore interesting from the point of view of the hosts, as well as for themselves. Much work from this viewpoint should be done. The BUFONIDÆ, LEPTODACTYLIDÆ and HYLIDÆ are closely related families. Of these the genera of BUFONIDÆ, other than *Bufo*, seem the most archaic. Their opalinids are wholly unknown. Anyone having specimens of any of these archaic bufonids should study their opalinids. This is the most important group of unexplored genera. But any unexplored anuran is likely to yield opalinids of interest, perhaps of very marked interest. Metcalf (1923) shows how geographic distribution of the hosts compared with the host-occurrence and geographic distribution of the parasites may be made very significant, and Metcalf (1929) reviews similar use of host-parasite data for all groups. Preserved specimens of ANURA, especially if preserved in alcohol, generally have their opalinids in good condition for study. Often the chromosomes can be counted in such specimens. Opening the specimens does not really injure them for museum purposes. There is thus here a great source of material waiting in many museums for study.

CHAPTER XXVII

BLOOD-INHABITING FLAGELLATES IN GENERAL

By

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The group of flagellated protozoa collectively referred to, though not with the strictest accuracy, as "Hemoflagellates" are placed in a single family, trypanosomidæ or trypanosomatidæ (Wenyon, 1926, and Calkins, 1926) under the simpler protomonads. They occur in vertebrate, invertebrate, and plant hosts. In the vertebrates they are found for the most part, though not exclusively, in the blood and other fluids of the body or in blood cells. In invertebrates the intestine is usually colonized though in some cases the infection is in the hemocele, in the salivary glands, or throughout the tissues. In plants various units of the latex system are infected.

There are four structural types of hemoflagellates as shown in Fig. 15. These four types represent generic groups in which species are placed because they manifest the particular structure of that group throughout the major part of their life cycle. In practically every species, however, the organisms change from their nomenclatorial type, some of them exhibiting all four structural stages during a life cycle. Thus the most complicated type, *Trypanosoma,* shown at "A" in Fig. 15, exists in vertebrate and invertebrate hosts. In the vertebrate host it appears usually in the trypanosome stage but in some cases is found in the leishmania stage (Fig. 15, D) ; in its invertebrate host, it is present in all of the four structural types. Members of the genus *Trypanosoma* are transmitted by an arthropod, a leech, or by contact. *Crithidia* (Fig. 15, B) is found only in invertebrates, in which it may occur in any one of the four stages. It is transmitted by cannibalism or by contamination, ingested cysts in the leishmania stage being infective. *Herpetomonas* (Fig. 15, C) is found in invertebrate and plant hosts and may occur in any of the four stages, the predominating one being herpetomonad. It is transmitted by the ingestion of leishmaniform cysts or, in the case of plant-to-insect and insect-to-plant transfer, by the ingestion or injection, respectively, of herpetomonad individuals. Members of the genus *Leishmania* occur in vertebrate and in invertebrate hosts. In vertebrates only the leishmaniform type is found; in invertebrates and in cultures, both herpetomonad and leishmaniform types are found.

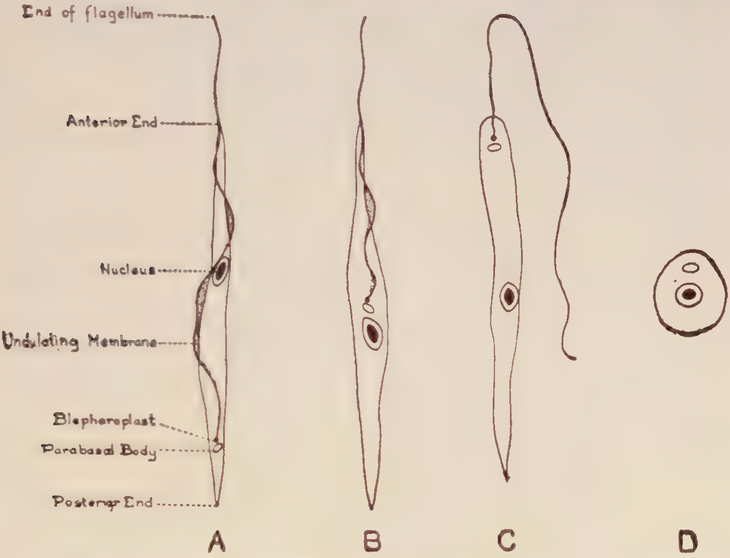

FIG. 15.—Comparative structure of the genera: A, *Trypanosoma* (*T. lewisi*); B, *Crithidia*; C, *Herpetomonas*; D, *Leishmania*. (After Hegner and Taliaferro.)

Three species of the genus *Leishmania* cause human diseases. Three species of the genus *Trypanosoma* cause disease in man, and a number of other species cause diseases in domestic and other lower animals. Consequently these forms have been the subject of considerable experimental study particularly regarding the chemotherapy of the diseases they produce, their mode of transmission, their restrictions as to host, cultivation, and reaction of their hosts to experimental infection.

The most available trypanosome for demonstration and study is *T. lewisi* found in wild rats. It can be readily subinoculated into white rats. The infection lasts as a rule for from thirty to a hundred days, after which the animal is immune to reinfection. Taliaferro and his collaborators (Taliaferro and Taliaferro, 1922; Taliaferro, 1923, 1924; Coventry, 1925) have clearly shown that the lack of pathogenicity of *T. lewisi* in the rat is due to the elaboration of at least two types of antibodies by the host, one of which destroys the parasites, the other inhibits their reproduction (*Cf.*, Chapter XL). The infection may be transferred from rat to rat by the intraperitoneal injection of trypanosome-containing blood, or, if desired, the transmission may be effected using the natural intermediate host, *Ceratophyllus fasciatus* (see Minchin and Thomson, 1915).

Various strains of trypanosomes pathogenic to rats and other small laboratory animals are carried on in a number of laboratories and are usually available to investigators who care to use them. These strains include species of *T. brucei, T. equiperdum, T. equinum, T. cruzi, T. rhodesiense,* and *T. gambiense*. Most of these species are quickly lethal to the rat. In the guinea-pig they cause a discontinuous type of infection where periods of remission alternate with periods of recidivation until, as a rule, the animal succumbs to the infection. In rabbits the course of the infection is similar to that in the guinea-pig except that it may be somewhat more prolonged and not as severe, the animals frequently recovering from the infection.

The species of *Crithidia* that are most available are the crithidial stage of *Trypanosoma melophagium,* which is found in the intestine of sheep keds, and *C. gerridis* from various species of the water strider, *Gerris*. The crithidial phase of *T. melophagium* is found in most specimens of the sheep ked, *Melophagus ovinus.* The parasite may be readily cultivated on Nöller's medium (N. N. N. medium plus glucose) from the intestine of the ked or from the blood of infected sheep. In either case, the crithidial phase predominates in the culture. Hoare (1923) has made the most thorough study of the life history of this parasite.

C. gerridis is found in water-striders throughout the length of the intestine below the anterior half of the stomach (Becker, 1923). It has not yet been cultivated, due to the difficulties of separating it from bacteria.

The herpetomonads found in the intestines of muscoid flies and of fleas are readily available. *H. muscæ-domesticæ* (*H. muscarum*) has been found in various species of *Musca, Phormia, Lucilia, Calliphora, Cochliomyia,* and *Sarcophaga. H. ctenocephali* has been found in the fleas of the dog and the cat, *Ctenocephalus canis,* and *C. felis,* respectively. These forms may be cultivated on N. N. N. medium though it is difficult to start the culture because of the bacterial contamination in the intestinal contents, particularly of the flies. The adult fleas which feed exclusively on blood have the habit of defecating as they near the end of a blood meal, and it is frequently possible to catch the dejecta upon a sterile cover-slip, and from this to transfer the organisms with a platinum loop to a tube of medium. The flies should be washed in a bactericidal fluid, and then immersed in sterile saline. Dissection should be made with sterile instruments. The intestine is carefully removed and is punctured near the esophageal end, care being taken not to enter the space within the peritrophic membrane. The fluid which exudes from the puncture will, on some occasions at least, be found to contain herpetomonads free from bacteria, and from this fluid cultures may be started on N. N. N. medium (Wenyon, 1926).

Leishmaniasis of any kind is not indigenous to the United States, and it is rare to find any cases. The few reported have been found for the most part in immigrants or sailors. Consequently most of the work done with *Leishmania* organisms has utilized cultural forms. All species seem to grow readily on N. N. N. medium or on Noguchi's serum medium (1924) which was devised for the cultivation of spirochetes (see Chap. XXIX, p. xxx). Strains of *L. tropica, L. braziliensis,* and *L. donovani* are available in a number of laboratories in the country.

CHAPTER XXVIII

THE INTESTINAL FLAGELLATES OF INSECTS

By

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THE numerous species which constitute the flagellate fauna of insects' intestines may be separated, for the sake of convenience, into four general groups.

First, there are the forms, many of them complex and rather bizarre, some of them cellulose-digesters, which live in the intestines of certain families of termites. One genus, *Lophomonas,* which shows close relationships with certain of the termite flagellates is found in roaches. Since the termite protozoa are treated in Chapter V, they will be entirely omitted from the present one.

Secondly, there are flagellates belonging to the family TRYPANOSOMIDÆ (Doflein) which spend a part of their life-cycle in the intestines of insects and the remainder in the blood stream or tissues of vertebrates. In such cases the insect is referred to as the intermediate host, but we must remember that in the natural order of things the flagellate is as much a parasite of the insect as of the vertebrate. In fact, there is abundant ground for believing that such flagellates have evolved from exclusively insect types. It is not inconceivable that some of the forms which we regard to-day as belonging to the genera *Herpetomonas* and *Crithidia* may in reality be phases in the insect of flagellates which have also a definitive vertebrate host. As an instance of this kind we could cite the case of the flagellate of the sheep ked which was supposed by many to be a *Crithidia* until Hoare (1923) showed experimentally that it was in reality *Trypanosoma melophagium* in its insect intermediate host. Other such cases of this kind should be looked for. What is the insect host, if any, of *Trypanosoma americanum* said to be common in American cattle? Is *Crithidia tabani*

Patton, 1908, in reality a trypanosome of cattle in its insect host? The flagellates definitely known to be trypanosomes, herpetomonads of the latex of plants or leishmanias will not be discussed further in this chapter, since they are involved either directly or indirectly in subjects treated in other chapters.

In the third group we recognize the members of the same family (TRYPANOSOMIDÆ) which, while they retain definite biological, structural, and phylogenetic relationships with the trypanosomes, phytomonads, and leishmanias, are, nevertheless, exclusively insect parasites with no definitive vertebrate or plant host.

To a fourth group we may consign all gut flagellates of insects belonging to families other than the TRYPANOSOMIDÆ—not such a motley assortment if we remember that their affiliations are strictly with genera which are preeminently intestinal flagellates of a wide variety of higher animals. The scope of the remaining portion of the chapter will be limited to these last two groups of gut flagellates of insects.

TRYPANOSOMIDÆ OF INSECTS

The TRYPANOSOMIDÆ of insects under consideration here comprise the now generally recognized genera *Leptomonas, Herpetomonas*, and *Crithidia*. These flagellates are primarily gut dwellers, although in certain cases other cavities and tissues of the insect's body are found to be occupied by them. In such cases the primary site of infection is the intestine. The number of described species is not only large, but constantly increasing.

One of the outstanding (not to say long-standing) problems is in connection with the validity of the generic names *Leptomonas* and *Herpetomonas*. Both were established by Kent (1880-1882) in the same volume. *Leptomonas bütschlii,* a flagellate found by Bütschli in the nematode, *Trilobus gracilis,* is the type of its genus. *Herpetomonas muscæ-domesticæ,* first observed by Burnett, is made the type of another genus. The former name has priority by two pages over the latter, and according to the rules should be retained if the two species can be shown to be cogeneric. A reinvestigation of the flagellate of the nematode is needed in order that we may know if its structure and life-history place it definitely in the same genus with the herpetomonads of insects. Bütschli's original paper described and figured a contractile vacuole in the anterior region of his flagellate, a cell structure unknown among the TRYPANOSOMIDÆ. In other respects, however, his figures would be considered representative also of the flagellates of insects, even to the "agglomeration rosettes." According to Doflein and Reichenow (1928), Goodey and Triffitt (1927) found a leptomonad in the nematode worm, *Diplogaster longicauda,* which developed a contractile vacuole when removed from its host.

The present practice (Wenyon, 1926, and Doflein and Reichenow, 1928) is to employ the generic name *Leptomonas* for TRYPANOSOMIDÆ which have only an invertebrate host and only two stages in their life-history, the leptomonad and the leishmania. *Herpetomonas* is the generic name for those in which additional trypanosome- and crithidia-like individuals may be found. We need to know if such individuals are not rarely present in species which are supposed to qualify for membership in the genus *Leptomonas*. The writer (1923) found two indisputably trypanomorphic specimens of *Crithidia gerridis* in one water-strider, as well as numerous leptomonas forms in the recta of a number of them. This is not to be expected in accordance with our orthodox conception of the genus *Crithidia,* in which such forms are not supposed to occur. To suggest that because such stages were found this flagellate is a *Herpetomonas* does not help, for the overwhelming preponderance of individuals were typical crithidias, the others merely incidental. Even in *Herpetomonas muscæ-domesticæ* the "leptomonad" forms predominate (although generally bi-flagellated due to precocious division).

The problems pointed out in the last two paragraphs are, then, more specifically these:

(1) Is *Leptomonas bütschlii* actually cogeneric with our insect flagellates which now bear the name?

(2) Can *Herpetomonas* and *Leptomonas* be separated on the basis of certain trypanosome- and crithidia-like individuals found in the former?

(3) May not these forms occasionally be found also in so-called true *Leptomonas* species? If so, is it not a relative matter not of generic significance?

Another problem, related to the above, is the significance of the trypanomorphic forms. The writer (1923) interpreted such stages in *H. muscæ-domesticæ* as flagellate forms in the process of encystment. He found, however, in *Muscina stabulans* and some other species of flies a different species with trypanosome-like forms which could not be thus interpreted. Likewise this process of encystment and the peculiar structure of the cyst as described by the writer for *H. muscæ-domesticæ* has not been confirmed in all respects. Are all rounded forms true cysts? Here are suggestions for further study.

The time has come for a general reinvestigation of the flagellates of insects with the problem of specificity in mind. It has been shown experimentally that one species of protozoön may establish itself naturally in the digestive tract of more than one host species. Conversely, in a number of cases it has been shown that one host species may harbor two or more species of one genus of intestinal protozoa. Experimental work has thus borne out the "principles" as set forth by Alexeieff. A number of problems present themselves.

The general problem of host-specificity, or the specificity of any particular flagellate for its insect host may be considered first. There is nothing of the nature of an unexplainable determinism about host-specificity. It may be defined as the adaptation of one species (the parasite, in the broad sense) to a complex of physical and chemical factors which obtain in (or on) another species or a more or less limited group of species (the host, or hosts). If the number of available host species is one, we say the parasite shows rigid host-specificity; if considerably restricted in suitable hosts we say that the specificity is limited; if the number of suitable hosts is comparatively large, or if they are fairly distantly separated taxonomically we speak of loose host-specificity. There is no parasite, however, which is not greatly limited in available hosts.

The writer (1923) and Drbohlav (1925) have shown that *Herpetomonas muscæ-domesticæ* can produce sustained infections in a number of genera and species of muscoid flies. This proves, apparently, that this flagellate may have more than one natural host, and that we cannot always give a parasite a new specific name simply because it is found in a host where it has not been previously noted. There are other herpetomonads and crithidias which need a similar reinvestigation, for example, the parasites in the SIPHONAPTERA in which group Wenyon (1926) lists sixteen species from which flagellates have been described. At least twelve different specific names are employed for these flagellates. This whole group deserves a reinvestigation involving a cytological study of the flagellates of each species and an ample series of cross-infection experiments. Whenever possible pure cultures should be made from each species of flea, and these cultures fed to the other species. Drbohlav (1925) has already paved the way for a start in this direction by working out the technique both for cultivating the flagellates and for handling the fleas. The experimental work should be correlated with careful cytological studies on the flagellates. Other groups can be investigated with the same general problem in mind; *e.g.,* the aquatic HEMIPTERA. Much still remains to be done in the DIPTERA. Wenyon (1926, pp. 1404-1414) has prepared a list of the known invertebrate hosts of the TRYPANOSOMIDÆ which can be consulted for suggestions as to the groups to be undertaken for the studies on host-specificity. One would infer from Drbohlav's experiments that cross-infection experiments of hosts considerably removed phylogenetically would give only negative results, but these results should not deter any worker from further attempts of this kind.

The converse of the problem just discussed is the specificity of the flagellates present in any one host. The writer (1923) has previously pointed out that more than one morphological type of *Herpetomonas* may be found in muscoid flies. *Herpetomonas muscæ-domesticæ*, the most numerous type, has the large parabasal body, often heart-shaped when dividing, prominent "marginal granules" and conspicuous basal granules. Another type, slightly shorter, has a smaller parabasal body, no marginal granules, and no apparent basal granules. A third shorter and broader type has still another type of parabasal body. There are probably other types which have been overlooked. These different types are probably of specific rank. It may be that Patton's *Rhynchoidomonas* is a valid genus. It is not unlikely that many of the muscoid flies will be found to have three or four species of flagellate parasites. Critical cytological and biometric studies will solve the problem. It would be extremely desirable to study cultures as well as insect infections. In this connection it should be pointed out that Chatton and his co-workers have identified about five species of TRYPANOSOMIDÆ from *Drosophila confusa.*

The problem of specificity of herpetomonads has been made to appear even more complicated by the work of Noguchi (1926). He applied, for the first time, extensive bacteriological technique, *i.e.,* agglutination, complement fixation, and carbohydrate-fermentation tests, as well as a study of the general morphology to the study of specificity of protozoa. A certain amount of work of the nature had already been done on trypanosomes and malaria organisms, but not in any such extensive and thorough fashion.

Referring again to the flagellates of muscoid flies, Noguchi (1926) found physiological differences between three strains of them. The flagellates *H. muscidarum* from *Musca domestica, H. media* and *H. parva* from *Calliphora* sp.? each reacted strongly with its own antisera in the agglutination and complement fixation tests, but not with the serum of another, thus proving the serological independence of the three strains. This physiological independence was further confirmed by the sugar fermentation tests. These three so-called species differed likewise in morphology. From a study of the plates one may recognize in *H. muscidarum* a typical *H. muscæ-domesticæ*, as described in detail by Prowazek (1904). *H. media* seems to be identical with *H. calliphoræ* Swellengrebel. Incidentally, it is possible that Wenyon (1913) was studying mixed infections of *H. muscæ-domesticæ* and *H. calliphoræ*. *H. parva* may possibly be the same species which the writer (1923) described and figured as one of three types. In fact, it appears that the three species figured by Noguchi are the same as the three types described and figured by the writer (1923, pp. 199-200). Noguchi's technique should be applied to the true *Herpetomonas muscæ-domesticæ* (as ascertained by cytological study) to see whether different strains of this type, as obtained from different species of flies, show identical or different physiological behavior.

It would perhaps be desirable to rework the muscoid fly flagellates first, but any one or more of other groups could be used in the same manner. The importance of correlating careful morphological studies with any and all experiments carried out must be stressed. The Schaudinn-iron-hematoxylin technique is a valuable one.

From the evidence submitted there appears to be ample reason for believing that more than one species of *Herpetomonas* is present in muscoid flies, and if future work confirms this belief, how shall the species be named? The writer (1923) and Hoare (1924) have reviewed the history of nomenclature of the flagellates of house-flies. We can be sure that Burnett did not apply any specific name. Leidy referred to fly flagellates, without describing them, as *Bodo muscarum*. Stein is responsible for the name *Cercomonas* *muscæ-domesticæ,* but he in turn gave no careful description. Prowazek (1904), however, gave an accurate description of one of the fly flagellates for which he retained the name *H. muscæ-domesticæ.* Therefore, should not this name be retained for the species to which it was applied, despite the argument of Hoare (1924) for *H. muscarum?* If it should develop that only one species of flagellate may be found in the "house-fly" (and it must be remembered that seventy-five years ago a "house-fly" might be any one of a number of species) there might be some argument for this procedure. Since Leidy gave no morphological description whatever of the flagellates he observed it seems doubtful if the specific name *muscarum* need be retained at all.

Any one who would desire to extend our general knowledge of this group (TRYPANOSOMIDÆ of insects) would probably have ample opportunity to describe new hosts and make studies of the parasites. A specimen of *Triatoma sanguisuga,* which occurs in the southern part of the United States, was sent to the writer a number of years ago. It was found to be heavily infected with a *Crithidia,* or the crithidial stage of a trypanosome. How do flagellates of flies "winter over" in our northern clime? These and a number of similar problems will confront workers investigating insect flagellates.

Under host-parasite relations we might discuss the problem of the effect of the flagellates on the health of their host. It is generally assumed that the host is not injured in the least by an infection of these teeming myriads of flagellates. Recently, however, Paillot (1927) has described a pathological flagellosis in the European corn borer (*Pyrausta nubilalis*) caused by *Leptomonas pyraustæ.* If this is true it is important, and more work is needed to either confirm or disprove this author's findings.

INSECT FLAGELLATES OTHER THAN TRYPANOSOMIDÆ

At the present time this group may not be so alluring to a prospective investigator as the preceding one. This is probably because not so much work of a fundamental nature has been done in this group, and hence the trend of future experimental work is not so well indicated. Comparatively few publications have appeared, and the number of recorded hosts and entozoic flagellates is not large. Among the more important papers are those by Mackinnon (1910, 1911) on flagellates of TRICHOPTERA and *Tipula* larvæ cited in the bibliography appended to this chapter. Some of the better known hosts and flagellates are as follows:

Host	*Flagellate*
1. TRICHOPTERA larvæ— (*Limnophilus rhombicus,* “ *flavicornis, Anabolia, Stenophylax, Sericostoma.*)	*Embadomonas agilis* *Eutrichomastix* (*Trichomastix*) *trichopteræ*
2. DIPTERA larvæ— (*Tipula* sp.?)	*Eutrichomastix* (*Trichomastix*) sp.? *Tetratrichomastix parisii* *Monocercomonas* sp.? *Polymastix* sp.? *Hexamitus* sp.? *Embadomonas agilis* *Embadomonas alexeieffi* *Rhizomastix gracilis*
3. COLEOPTERA—	
Passalus sp.? (adult)	*Eutrichomastix* (*Trichomastix*) *passali.*
Melolontha vulgaris (larva)	*Polymastix melolonthæ* *Monocercomonas melolonthæ*
Cetonia sp.?	*Monocercomonas cetoniæ* *Polymastix* sp.?
Oryctes nasicornis (larva)	*Polymastix* sp.? *Monocercomonas* sp.?
4. ORTHOPTERA, family BLATTIDÆ—	*Lophomonas* (several species) *Monocercomonas orthopterorum*

This list was rather hastily compiled, and is probably incomplete, but a number of problems are suggested. *Melolontha, Cetonia,* and *Oryctes* are all representatives of the beetle family SCARABÆIDÆ found in regions other than North America. What flagellates are found, if any, in our American scarabæids? May not other families of beetles include hosts of flagellates of the genera *Monocercomonas* and *Polymastix?* Then, also, the question of specificity and host-specificity of the flagellate comes up here again as in the TRYPANOSOMIDÆ. Comparative cytological studies on the flagellates of different hosts and cross-infection experiments may be in order.

The writer has examined and made a few slides of flagellates from the large gut of tipulid larvæ taken from a small stream near Lake Roland, Baltimore. Most of the flagellates described by Mackinnon except *Rhizomastix* were observed. There is now an opportunity for someone to carefully study and publish on the flagellates in our American tipulids. To the author's knowledge no one on this continent has yet examined trichopterous larvæ for flagellates, although such larvæ are usually easily obtained.

Another problem is suggested in a statement by Mackinnon in which she expresses her belief that the flagellate *Rhizomastix,* which she found in the larva of *Tipula,* a crane-fly, is the same as that which had been found by Alexeieff in the intestine of an axolotl. Here is a problem. Can insect flagellates be introduced into cold-blooded vertebrates? And the converse—can the flagellates of cold-blooded vertebrates develop in these aquatic larvæ (*Tipula* and TRICHOPTERA)?

Thus far only one flagellate of this group, *Trichomastix passali,* has been cultivated, and that by Tanabe (1926). Efforts should be made to cultivate the others. The flagellates of the crane-fly larva would be a good starting place because so many species are to be found in this insect.

CHAPTER XXIX

PROTOZOA OF LATEX PLANTS

By

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INTRODUCTION

Nature of latex protozoa. The PROTOZOA of latex plants have been studied in many countries since their first discovery. In all cases the organisms have been members of the hemoflagellate genus *Herpetomonas*. These latex herpetomonads are insect protozoa capable of living in the extensive duct-like vacuoles in the latex cells of milky-juiced plants on which their insect hosts are accustomed to feed, just as trypanosomes are typical insect protozoa which spend part of their life-cycle in the blood streams of higher animals. Latex is not like animal blood, however, but is the secretion which certain long, branching, multinucleate cells of latex plants pour into vacuoles in their cytoplasm. These vacuoles are continuous, having fused to form an extensive channel within each laticiferous cell except near the growing tips of the plants. The latex does not circulate. Types of latex vary as to dominant constituents, but in general there are present resins, fats, wax, caoutchouc, tannin, proteins, alkaloids, mineral salts and sugars in varying amounts. Within the plant the latex is sterile except when the specialized latex herpetomonads have been introduced with the salivary fluid of plant-feeding insects.

Latex flagellates live in the latex itself, that is, in the milky contents of the vacuoles of the latex cells. They can neither penetrate the adjacent cytoplasm, from which they are separated by an extremely thin membrane, nor leave the cell vacuole in which they find themselves to enter the vacuole of an adjacent cell. Once forced from the salivary glands of an insect into the vacuole of a given latex cell of a suitable host plant a latex flagellate has no alternative to living within this single cell and to multiplying in it indefinitely. Its progeny may invade and colonize the whole length of the cell, including all of its branches, to the growing points where the latex vacuoles are not yet developed. As the cell grows the flagellates may progress with it, invading the new portions as they are formed in the extending tissue, until the progeny of the flagellate originally introduced into a plant may find themselves many feet away from the site of the insect inoculation. Under laboratory conditions the originally infected portion of an old plant may be cut away and the new growth furnished with roots by regeneration, with the result that the flagellates continue in the new portions of the original cell. The flagellates introduced into latex plants would die with the death of the latex cell and be unable to function in the preservation of the species were it not for the fact that the insect hosts in feeding on the plants occasionally suck up latex and flagellates simultaneously, thus completing the cycle.

Families of plants infected. Latex occurs in the plant families EUPHORBIACEÆ, ASCLEPIADACEÆ, MORACEÆ, URTICACEÆ, APOCYNACEÆ and COMPOSITÆ in so many species as to be a well-known characteristic of these plants. In many other families latex is found in occasional species. Latex flagellates have been carefully studied in plants of the EUPHORBIACEÆ and of the ASCLEPIADACEÆ, and there have been reports of similar organisms in plants of the genus *Ficus* in the MORACEÆ. Lists of host plants have been given by Lafont (1910), Nieschulz (1925), Gaschen (1926) and Wenyon (1926).

In the plants of the EUPHORBIACEÆ and ASCLEPIADACEÆ the type of latex system is that known as simple or unfused. In such a system the latex cells never join during their growth, and flagellates present in one cell do not have access to neighboring cells. In other plants, as in the COMPOSITÆ, the latex system is of the fused type, in which the latex cells of the entire plant are joined together by frequent fusions of neighboring cells. Latex flagellates have not yet been found in plants with such fused latex cells, but if they should be introduced into any part of such a system they would probably be able to penetrate the whole. It will be of interest to find what phenomena attend infection if such plants are found to contain protozoa in their latex.

History of discovery. Herpetomonad hemoflagellates were first discovered in the milky juices of plants by Lafont in Mauritius in the year 1909. Similar flagellates were soon described by other investigators throughout the tropics and subtropics. This made it evident that herpetomonads inhabit the latex cells of many plants as characteristically as other herpetomonads inhabit the intestinal tracts of insects. These protozoa of plants depend upon insects to carry them from plant to plant, and when in the insects they multiply in the intestinal tract and in the salivary glands.

The flagellate species first reported from Mauritius by Lafont in 1909 was given the name *Leptomonas davidi* Lafont. It was found in the latex of *Euphorbia pilulifera* by his assistant, David, whom he had asked to examine the milky juices of these plants to discover whether they might be good culture media. After 1909 reports of the presence of latex flagellates came from India, Reunion, Madagascar, Mayotte, Zanzibar, Dahomey, Upper Senegal and Niger, Martinique, Portugal, Belgian Congo and New Caledonia. Later reports came from Sardinia, Italy, Paraguay, and Venezuela. Recently reports have come from France, Switzerland, Algeria, Uruguay, Alsace, Honduras, the United States of America, Central America, Roumania, Brazil, South Africa, and Queensland.

A map showing this very nearly world-wide distribution of latex flagellates is shown in Fig. 16. As the map indicates, certain portions of the world have been more thoroughly investigated than others. The islands of the Indian Ocean, much of Africa, the countries of southern Europe and the eastern coast of North and South America have been examined to some extent; in a few localities extensive studies have been carried on. Some portions of the world, notably the countries of the Far East, the southern United States, Mexico, and parts of South America have received little attention. There are few locations within the indicated general range in which a careful survey has failed to bring to light some species. The uninvestigated regions in the tropics and subtropics are probably furnished with abundant instances of latex plant infections by protozoa of this group. It remains only for investigation to be made in these countries to bring to the attention of the world new species of latex protozoa, new insect hosts and new plant hosts in considerable numbers. It is to be hoped that investigators who may be able to survey these regions in the near future will make use of the recently developed methods of research for carefully characterizing the species of organisms found, so that descriptions in the growing protozoölogical literature may be accurate and complete.

Morphology and classification of latex protozoa. The protozoa thus far discovered and carefully studied in latex plants have all proved to be typical herpetomonads. A chapter could be written on the other types of organisms reported from latex, but little evidence has been presented to prove the real nature of these forms. Bacteria, spirochætes, amœbæ, and trypanosomes are supposed by some to be found occasionally in milky-juiced plants. A single brief paper (Picado, C. R., *Comptes rendus de la Société de Biologie,* 84: 552, 1921) discusses the presence of a bacterial infection in a latex plant. Spirochætes have been reported as occurring in latex plants (Laveran and Franchini, 1921), but more intensive studies are needed of any cases in which they may be found. Amœbæ have also been reported (Franchini, G., *Bulletin de la Société de Pathologie Exotique,* 15: 197-203, 1922), but have not been fully described. Trypanosomes and trypanosome-like organisms have been mentioned (Franchini, G., *Bulletin de la Société de Pathologie Exotique,* 15: 18-23, 1922). The normal nuclei of the latex cells of the plants stream out with the latex when young leaves or stems are broken. They have been taken

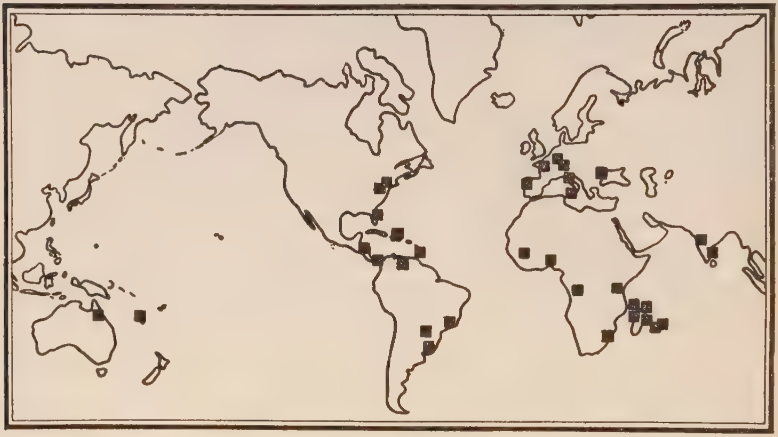

FIG. 16.—Map showing the locations from which reports of latex flagellates have come.

for microörganisms and described accurately by DuPorte (*Journal of Parasitology,* 11:183-194), who so carefully pictured them that they can be identified beyond reasonable doubt. Bruni (1925) also pictures cell nuclei as well as herpetomonads, confusing the two. In Fig. 17 A, latex cell nuclei are shown in the presence of herpetomonad flagellates. Thoroughly studied cases of herpetomonads of latex plants have frequently shown a few *Leishmania*-like forms. These have not yet been proven to have existed as intracytoplasmic parasites in the usual sense of the term, but have been found with the herpetomonads in the latex, insect intestinal contents, or cultures. These *Leishmania*-like forms may represent small rounded herpetomonads rather than definite stages in the life-history of the flagellates. Whenever accounts of latex protozoa other than herpetomonads are presented extensive proofs should be furnished for the claims as to the nature of the forms under observation.

It is probable that a very large number of species of true herpetomonads exist in latex plants. Only six species have been named, however. The species *Herpetomonas davidi* (Lafont) has been the subject of extensive research. *Euphorbia* flagellates from all parts of the world have been assigned to this species. It is possible that in some cases the organisms observed belonged to other closely related species. *Herpetomonas elmassiani* (Migone) and *Herpetomonas lygæorum* Noguchi were distinguished accurately by Noguchi, whose work with these protozoa and some insect flagellates and leishmanias established the possibility of identifying such organisms with other strains of the same species even in cases derived from alternate hosts, and to distinguishing them from other species even when morphologically similar. *Herpetomonas bordasi* (França) has not been studied extensively, but is well characterized by its original description. *Herpetomonas ficuum* was described by Fantham in 1925, but has not been studied by others. A flagellate has recently been reported from a fig species by Bancroft in Queensland. *Herpetomonas françai* was described by Aragao in 1927 in a species of *Maniot* in Brazil.

Insects which serve as alternate hosts. All latex flagellates, so far as known, are dependent upon insect hosts for transfer from one plant to another.

Herpetomonas davidi is reported as being transmitted by *Stenocephalus agilis* in Portugal (França, 1920), by *Dieuches* *humilis* in Dahomey (Bouet and Roubaud, 1911), and by *Nysius euphorbiæ* in Mauritius (Lafont, 1911*a*).

Herpetomonas elmassiani is transmitted by *Oncopeltus fasciatus,* the eggs, nymphs and adults of which are shown in Fig. 17 B and by other species of the same genus. The insect host of *Herpetomonas lygæorum* is probably *Lygæus kalmii,* since the species has been collected in this insect (Noguchi, 1926).

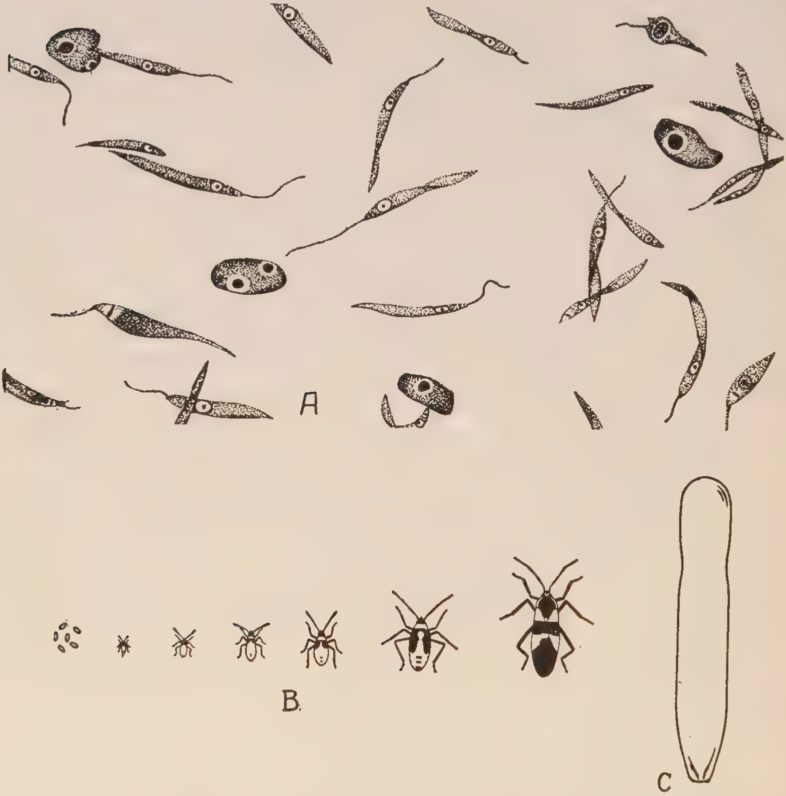

Fig. 17.—A, Latex flagellates of the species *Herpetomonas elmassiani* Migone in the presence of several nuclei of the latex cells of the host. The latex cell nuclei are readily recognized by the large spherical nucleoli, each of which is surrounded by a halo. The flagellates each contain a single nucleus of delicate construction, and a rod-shaped parabasal body. B, Eggs, first, second, third, fourth and fifth instar nymphs and adult of the hemipterous insect *Oncopeltus fasciatus,* the insect host of the latex flagellate *Herpetomonas elmassiani.* C, Diagrammatic representation of water tube used to maintain a constant supply of water for insect colonies.

The insect transmitters in other cases are unknown, but will probably be found to be members of the insect family LYGÆIDÆ, to which all of the above species belong. The LYGÆIDÆ all feed upon plants, and some of them have been known previously as hosts of hemoflagellates.

SPECIAL METHODS OF STUDY

Recently devised methods have made it easy to identify and work with the protozoa of latex plants. The following procedures have to do with the maintenance of such forms under laboratory conditions in cultures and in plant and insect hosts, as well as with methods of staining and examining the organisms from these sources.

Cultivation of latex flagellates. Cultures of latex flagellates are difficult to establish. Suitable media may be inoculated with the organisms many times without the formation of successful colonies. Yet when multiplication begins under these artificial conditions divisions are rapid and great numbers of new individuals appear.

Franchini (1923) and Nieschulz (1924*a*) used plates of Nöller's horse blood agar in the cultivation of a flagellate from a plant of *Euphorbia cerciformis* which grew in the Botanical Gardens at Bologna. Nöller's medium is a nutrient agar, containing one per cent of agar and one per cent of glucose, to which an equal part of defibrinated horse blood is added before pouring plates.

Migone (1922) in Paraguay used a human blood agar to cultivate *Herpetomonas bordasi* (França) from the host plant *Morrenia odorata.*

Noguchi (1926) in the United States succeeded after many attempts in establishing seven strains of latex herpetomonads on his leptospira medium which has the following composition:

NOGUCHI'S LEPTOSPIRA MEDIUM

0.9% sodium chloride	800 parts
Fresh rabbit serum	100 parts
2% nutrient agar	100 parts
Rabbit hemoglobin	10 parts

The rabbit hemoglobin is made by laking one part of defibrinated blood with three parts of distilled water.

By the use of cultures Noguchi (1926*a*) devised two important methods of identifying species of hemoflagellates. In cultures in which the flagellates had become established, various sugars were added to the leptospira medium. The protozoa were able to ferment some, but not all of the sugars. A given species was definitely characterized by the sugars it was able to utilize. Thus *Herpetomonas elmassiani* in cultures derived from the insect hosts from North and South America and from the plant host from North America was able to ferment thirteen sugars and other carbohydrates with acid formation. These were glucose, levulose, mannose, saccharose, raffinose, inulin, galactose, maltose, salicin, zylose, mannitol, dextrin, and arabinose. It did not attack amygdalin, lactose, dulcitol or rhamnose. On the other hand, *Herpetomonas lygæorum,* although found at times in the same plant and insect hosts, was able to ferment only glucose, levulose, and mannose, and none of the other fourteen compounds.

Another method of identification was established by means of such cultures. Noguchi injected a rabbit on four occasions at intervals of four days with one to two cubic centimeters of a rich culture. On the ninth day after the last injection he secured an immune serum. When 0.05 of one cubic centimeter of this was added to one cubic centimeter of a culture of the same species of flagellate the cells in the culture swelled and burst, thus causing clearing of the medium and the formation of a layer of whitish sediment. The serum was effective against all strains of the species, even when derived from the alternate hosts. A variety of other hemoflagellate species in culture were tested with these immune sera, but no relationship was indicated between the latex protozoa and other groups, although there was a slight reaction of each of the two latex flagellate species tested to the immune serum derived from the other one.

These methods of species identification will prove valuable in the future in the discovery of insect vectors of hemoflagellates, since forms in insects can be definitely established as identical with or different from strains found in infections under consideration.

Even with media in which the latex flagellates multiply, the isolation of new strains from plants is difficult. Often the organisms die without beginning to divide to form new individuals. The transfer from a successful culture to new tubes of culture medium is very uniformly successful, however. The sterilized medium described below is suitable for subcultures from the blood culture media.

YEAST BROTH

Bread yeast (Fleischmann's compressed yeast)	5.0 gms.
Water	95.0 gms.
Sodium chloride	0.5 gm.
Glucose	1.0 gm.

Sterilize twenty minutes at fifteen pounds pressure in a steam autoclave. The sugar may be omitted but is desirable because it promotes vigorous growth.

Cultures maintained in this readily prepared medium are of interest because the organisms are not given blood as a constituent of their food supply, and because the medium may be sterilized by heat.

Maintenance in plant hosts. A specialized technique is involved in the maintenance of latex flagellates in their plant hosts under laboratory and greenhouse conditions. *Herpetomonas elmassiani* (Migone) occurs in the field in *Asclepias syriaca* as far north as New Jersey on the Atlantic Coast. Since this plant is unsuited for greenhouse use on account of winter dormancy, a better host plant for experimental work is *Asclepias curassavica,* a species used in this country as an annual ornamental, seeds of which can be obtained readily from seedsmen. In the greenhouse this plant lives many years. Plants grown from seed are fortunately free from flagellates even when the seed is derived from infected plants. This is explained by histological examinations which show that there is never a connection between the old latex cells of the parent plant and the tissues of the seed. The seedlings are easily infected by allowing infected insects of the genus *Oncopeltus* to feed on them. About two weeks after an infective feeding flagellates begin to be conspicuous in the latex, though they may be found in small numbers at the end of one week. The plants will remain infected indefinitely if given proper care. When the growth becomes too tall each large plant may be used as a source of several smaller ones by making cuttings and rooting these. The stem is cut with a sharp knife into sticks about six inches in length; a few leaves are allowed to remain near the top of each cutting. The pieces of stem are placed in sand so that they are two-thirds covered, the leaves being near the surface of the rooting medium. The sand is moistened regularly to avoid uneven soaking or drying until new roots form. In four weeks most of the cuttings form roots. Then the new plants are transferred to soil in pots; when they are established a microscopical examination will show them to be well infected in most instances. If the infection in the original plant was light in the sense that few of the existing latex cells had been infected by insects, some of the cuttings will send out shoots from axillary buds of the stem from which latex flagellates may be entirely absent. This results from the failure of the old infected latex systems to penetrate the new tissues. Such cuttings must be discarded, and in the later growth of each plant branches which show no flagellates in their leaves should be trimmed away. If care is taken to confirm the presence of the flagellates from time to time by microscopical examinations and to remove branches from which they are absent it is possible to maintain infections in these laboratory plants for years. *Asclepias nivea* and hybrids between *Asclepias curassavica* and *nivea* may be used for the same purpose and handled in the same way with success.

Maintenance in insect hosts. As has been suggested above, it is necessary to maintain colonies of the insect hosts of the flagellates in order to secure infection of seedlings when desired. This is done easily for *Oncopeltus fasciatus,* and probably for other species in the same genus, by means of the following technique:

During late summer the insects are collected in the field. They may be confined in Erlenmeyer flasks or other laboratory glass containers, for crowding in small jars does not seem to be harmful if food is supplied properly. Dry *Asclepias* seeds serve as food; such seeds are easily collected in the fall from native species. When fed on the foliage of plants in the greenhouse many insects die, but they thrive on seeds. Fresh drinking water must be provided in abundance. This is best furnished in inverted tubes, each with a small opening at the lower end; for this purpose test tubes may be melted into the shape shown in Fig. 17 C and hung from the side of the container by a wire or cord.

Insects collected in the field may or may not be infected with flagellates. To secure an uninfected stock a small amount of cotton is placed in the bottom of the colony jar, where yellow egg masses are soon hidden by the fertile females. Before the eggs become orange in color they are removed from the presence of adults to fresh colony jars. Nymphs hatching from these eggs are free of flagellates, ensuring a stock of uninfected insects which can be maintained on seeds and water indefinitely.

If a colony of infected insects is desired, a number of individuals are isolated and fed on infected plants of *Asclepias* for a few days. Some of them will become infected from this feeding; but if it is desired to infect all of the individuals prolonged feeding on infected plants may be necessary.

Dry seeds are better food material than moist seeds because they remain in perfect condition for weeks without sprouting or rotting as wet seeds tend to do. The insects seem to be able to utilize moist or dry seeds equally well. In feeding, the insects place their beaks in the seeds and remain quietly in position for some minutes, finally pumping vigorously. Apparently they digest their food before ingesting it, a process for which the large amount of saliva present in their salivary glands must be useful. The same process has been watched when an adult has discovered an egg and attempted to suck it. First the beak is carefully adjusted within the egg. Then a period of several minutes intervenes in which the egg is entirely normal in appearance. Presumably during this period it is being digested by the salivary fluids. Suddenly the insect becomes restless, shifts its position slightly, begins a pumping motion, and within a few seconds empties the entire egg, which changes from its completely normal appearance to that of a thin papery shell, its hitherto turgid walls collapsing rapidly. The large amount of saliva needed for this process of feeding may account for the need shown for a constant supply of water. If water is not readily available the insects sometimes kill and suck each other. It is therefore important to maintain an adequate supply. If a colony has been deprived of water for a few hours the insects become flattened in shape and dull in color. When water is supplied they drink greedily and soon expand, becoming glossy individuals again. The water tube described above serves this purpose well. The insects find the small opening and congregate around it to drink.

Staining latex protozoa. Another special method in the study of latex flagellates is that of fixing and staining microscopical preparations. Latex flagellates are distorted by drying in thin smears, and if dried must usually be examined in thick smears. The most convenient way to detect flagellates in latex samples from large numbers of plants in the field is to use a quick stain which is not sufficient for cytological study, but is useful for preliminary work. Many smears of latex can be collected in the field on a single glass slide. On each slide ten or twenty short straight-line smears parallel to each other may be drawn by using the cut petioles of leaves from separate plants. After the smears dry, the slides are wrapped in paper for transport to the laboratory where fixation is accomplished by immersion in absolute alcohol. Subsequent staining for ten seconds with a few drops of the ordinary anilin gentian violet solution used for bacteria suffices to show the presence of flagellates. The excess of stain is removed by running tap water over the slide, which is then drained. This stain is very quickly applied to large numbers of smears and the flagellates are rendered conspicuous by its use, since it stains the flagella intensely. Smears from infected plants can be distinguished from those from uninfected specimens by the use of a four millimeter or even a sixteen millimeter objective, and a 10x ocular.

For more careful staining of dried smears Wright's blood stain is used. This staining mixture contains a fixing agent and so may be placed directly on the dried slide, allowed to remain there for one minute, then diluted with an equal part of distilled water. The diluted mixture forms a metallic film on its surface, and is allowed to stand two minutes, after which the slide is rinsed in distilled water and drained. This method of staining differentiates the organelles very well. The nuclei stain pink, the parabasal bodies deep blue, and the cytoplasm pale blue. The flagella stain with difficulty but appear purplish when visible. If the flagella are not well stained a small portion of a smear may be treated with a drop of anilin gentian violet in addition to the original Wright's stain, thus making the flagella conspicuous on the individuals in part of the sample. This is particularly necessary if measurements are to be made of the total lengths. Field survey slides stained originally with anilin gentian violet, if desired for more careful examination in the laboratory, may be restained with Wright's mixture after destaining with several changes of methyl alcohol.

For careful study of the finer structures of the flagellates it is necessary to fix them while they are still in moist latex. This may be done by dipping the fresh smears into absolute methyl alcohol, Schaudinn's fluid, Regaud's fluid or some other standard fixing solution. The slides must be immersed promptly to avoid drying at the edges of the smears where the fluid layer is thin. After thorough fixation, and the removal of the fixing agents, the slides may be stained as though they held sections of tissues, using iron hematoxylin, Delafield's hematoxylin, Giemsa's stain, or other standard stains.

PROBLEMS FOR INVESTIGATION

Effect of latex protozoa on the health of the host plants. Perhaps the most important question which presents itself is that of the effect of latex protozoa on the health of the host plant. Lafont, the original discoverer of latex flagellates in *Euphorbia pilulifera,* believed that the infected plants were noticeably harmed by the presence of the organisms. In this view some later workers concurred; these were França (1911), who worked with *E. segetalis,* and Gaschen (1926), who studied *E. cyparissias* and *E. gerardiana.* It will be noticed that these investigators worked with flagellates in plants of the genus *Euphorbia.* No report of injury to one of the ASCLEPIADACEÆ has been published. A considerable number of workers reported that the infected euphorbias with which they worked showed no macroscopic symptoms distinguishing them from uninfected plants of the same species; among these investigators were Leger (1911) who worked with *E. pilulifera,* Bouet and Roubaud (1911) with the same plant, França (1911) with *E. segetalis,* Rodhain and Bequaert (1911) with *E. indica,* Noc and Stevenel (1911) with *E. pilulifera,* and Aubertot (1927) with *E. cyparissias.*

To avoid errors in such work a number of precautions must be taken. Injury due to transplanting or cultivating under unfavorable conditions must be avoided. Many of the hosts of latex flagellates are weeds not commonly cultivated. Such wild plants do not always respond favorably to the treatment given to garden species. A sufficient number of uninfected plants must therefore be maintained as controls to show that the experimenter has been successful in handling the host plant itself and in protecting it from injuries due to abnormal environmental conditions other than the flagellate infection. Excessive insect injury due to the feeding of the insect host of the flagellates must be differentiated from possible injury caused by the protozoa themselves. Thus in infecting species of *Asclepias* by means of *Oncopeltus fasciatus* infected with *Herpetomonas elmassiani* an excessive number of insects on a young plant or on the tender growing shoot of an older plant sometimes causes wilting and even death of the top of the plant.

Such plants recover, however, and appear healthy by the time flagellates become numerous in their latex. Similar plants infected equally heavily by a single insect may show no injury at all.

Seasonal changes in the host plant may be confused with the effect of flagellates if careful observations are not made. In *Asclepias syriaca* under the conditions in the United States (Holmes, 1925*a*) the presence of the non-pathogenic species *Herpetomonas elmassiani* has been found to bear a relation to yellowing and ripening changes in the host plant after seed production in the fall. This does not indicate that the organisms are the cause of the yellowing. Plants which fail to blossom appear green and vegetative, and are not found to contain flagellates. Yet such immature plants may be infected by confining the insects on them, and when so infected remain green and vegetative as before. Experiments and observations have shown that the flagellates are absent from the green plants because the insect host prefers to feed on plants which have blossoms and fruit. The yellowing and ripening changes in these older plants, however, are independent of the presence of the flagellates and depend wholly on the normal processes accompanying seed formation in the host plant.

By means of such observations it will be necessary to determine for each species of latex flagellate discovered in the future its relation to the health of its host plant. Some of the flagellates of *Euphorbias* already studied need more careful work of this sort. Climate, seasonal changes and other environmental factors may be the causes of the differences of opinion, but they must be carefully studied before being considered the responsible factors.

Effect of flagellates on the health of the insect hosts. Thus far no observations have shown the exact effect of the presence of latex flagellates in their insect hosts upon the health of the insects. But it is possible that injury may occur in some cases. One difficulty in conducting studies of incubation periods in the insect host is the frequent death of insects before they become infective. These deaths are numerous at the time when the insects would be expected to become infective. Careful examination should be made to see whether there may be intracellular stages of the flagellates at this time, and corresponding damage to tissues preceding entrance into the salivary glands. Little is known about this, but methods are available for a careful study of the progress of the infection in the insect host. Since the insects can be reared in some cases in enormous numbers in small laboratory containers and secured free of the infection by being thus hatched from the egg in the absence of older insects, and since it is only necessary to feed such insects on infected plants from greenhouse stocks or cut tops of infected plants from field collections to obtain material for study, it should be possible to obtain the required information.

Information on the length of the incubation period, or time after the first infective feeding preceding the first possible transmission to plant hosts, is needed for all the plant flagellate species. This period must be correlated with the progress of the infection within the insect.

Specificity for insect hosts. It seems probable that more than one insect species can act as vector for a given latex flagellate. Thus *Herpetomonas davidi* has been reported as carried by *Stenocephalus agilis*, *Dieuches humilis* and *Nysius euphorbiæ* as previously stated. The identity of the flagellates in these hosts should be confirmed. In the writer's experiments *Oncopeltus fasciatus* has repeatedly transmitted *Herpetomonas elmassiani* to seedling host plants in greenhouses in a region where no transmission occurs outdoors. *Oncopeltus cingulifer* (Noguchi, 1924), *O. luctuosus* (França, 1921), *O. quadriguttatus* (Bancroft, 1928) have been found associated with infected plants of the ASCLEPIADACEÆ. It will be necessary to establish colonies of each insect species, rear nymphs from eggs and infect them from known sources of flagellates, subsequently infecting healthy seedling plants by allowing these insects to feed on them, in order to prove that they are capable of acting as transmitters.

It has been observed that insects of the genus *Lygæus*, close relatives of *Oncopeltus*, can take up the flagellate *Herpetomonas elmassiani* from infected *Asclepias* plants, and that the flagellate appears in the feces in twenty-four hours. Infected specimens of *Lygæus kalmii* were found in the field by Noguchi. But the geographical distribution of the plant infections makes it appear probable that *Lygæus* is not associated with the spread of this flagellate. Experimental investigation is needed. A considerable number of species of *Lygæus* can be obtained. The *Catalogue of the Hemiptera of America North of Mexico*, University of California Publication in Entomology No. 2, by Van Duzee, 1919, lists the species native to each locality in the United States.

The full life cycles in insects are not known in most cases. *Herpetomonas davidi* has been traced through *Stenocephalus agilis* by França. It is still a question whether latex flagellates are intracellular at any stage in the insect hosts.

Flagellates show abnormal methods of division in hosts not normal to them. Thus *Herpetomonas elmassiani* in *Lygæus kalmii* forms small bud-like aflagellate cells at the anterior end of the organism, clustering around the flagellum. These have been observed in the living condition. When stained such forms each show a nucleus and a parabasal body, but no new flagellum. They are very short, and are present to the number of two or three or more at the anterior end of the parent cell. It would be desirable to know what their nature and function are.

Degree of specificity for plant hosts. Latex flagellates are not specific for individual species of plants. By means of *Oncopeltus fasciatus* the writer has transmitted *Herpetomonas elmassiani* to several *Asclepias* species, *A. nivea, A. curassavica, A. pulchra,* and *A. incarnata,* and from all reports the flagellate may be transmitted to any species of the family ASCLEPIADACEÆ. The range of *Herpetomonas davidi* is considered to be very wide, but only field observations are available. A list of *Euphorbia* species in which this flagellate has been found is given by Gaschen (1926), and by Wenyon (1926).

Possible presence of protozoa in commercial latex plants. Since most of the commercially valuable species of latex plants are cultivated in warm climates where insects with flagellate infections are abundant, it is probable that flagellates will be found in some cases when adequate search is made. Such search has not yet been made in commercial plantings of figs or rubber trees. It is probable that the now extensive plantations will eventually attract HEMIPTERA capable of feeding upon the cultivated species. These HEMIPTERA may then introduce protozoa formerly confined to small numbers of wild plants in the neighborhood.

Preliminary statements by Fantham in South Africa and Bancroft in Queensland mention latex flagellates in *Ficus* species (*F. edulis* and *F. scabra*). It is not yet known whether the organisms are harmful to the hosts. In commercial rubber plants no flagellates have been reported, and in other latex plants no statements have been published as to the effect of the presence of the flagellates on the rubber content of the plants.

The identification of species. In the absence of adequate criteria and careful tests flagellate species may be wrongly or uncertainly identified with the result that the information gained cannot be used by others. It is therefore desirable that the serum tests previously described be used whenever it is necessary to learn whether two strains of flagellates are specifically identical or not, and that sugar fermentation tests be used when it is desired to characterize a new species or refer a recognized one to its proper name. Fortunately flagellates react to these tests in their characteristic manner without reference to the particular host in which they have last grown. For this reason it is possible to identify the strain in an insect with a strain of the same species derived from a plant host without performing transmission experiments.

It is probable that the careful use of such tests on strains of flagellates formerly reported as *Herpetomonas davidi* will show that more than one species is represented, since it is probable that the extensive geographical range now ascribed to this species contains many species of flagellates to correspond with the numerous insect species present.

Methods of overwintering. In the warmer regions of the world infections in plants and in insects continue throughout the year. An example of this is given by Migone (1916), who states that *Araujia angustifolia* loses its leaves in winter but retains the flagellates in the latex of the stems and twigs. In cooler regions both hosts have long rest periods during the winter months. It is of great practical importance to know how the flagellates pass the cold season. França found that *Herpetomonas davidi* overwinters in the salivary glands of dormant adult insects or large nymphs, and also in the latex of the stems of *Euphorbia* plants.

In the case of *Herpetomonas elmassiani* in the northern part of its range in the United States the preliminary evidence points to overwintering in the latex in small portions of the basal part of the stem, which alone retains the latex after the death of the stem above ground. Latex cells in this basal portion of the stem may penetrate the new growth the following year. It is desirable that infections be studied before the plants begin growth in the spring. By marking the location of heavy infestations in the fall it may be possible for some worker who has infected plots at hand to work out this problem. The overwintering stage of the insect host, *Oncopeltus fasciatus,* is not known.

The possibility of inoculating without the aid of insects. Another problem which has not been settled is the question whether it is possible to inoculate plants with the latex flagellates without the aid of insects. Attempts have been made by introducing flagellates in fine glass pipettes into the petioles and stems of uninfected plants, and by dipping thread in infected latex and then pulling this with a needle through uninfected plant parts. There have been a few claims of success with these methods, although reports of failures have been more numerous. The writer has tried in these and other ways with *Herpetomonas elmassiani* in *Asclepias* species, but has never obtained transfer of the flagellates to the uninfected plants in spite of persistent attempts.

França (1914) reported three successes in many trials, two with the pipette method and one with the thread dipped in infected latex. Noc and Stévenel (1911) also report successful transfers in two cases. In none of the successful cases were the uninfected plants grown from seeds by the experimenters. Because of this the possibility that infections existed but were not discovered in examinations made before each experiment must be taken into consideration. Rodhain and Bequaert (1911) report that two days after attempts at transfer the flagellates swarmed in the experimental plants. This seems to point to an earlier infection since the result of insect infection studies would indicate that more than a week would be required for an infection to become so general.

The difficulties met in forcing latex flagellates into broken latex cells of uninfected plants seem to be due to several factors, namely: (1) The latex in the uninfected cells is under pressure and tends to sweep infected latex away from the uninfected cells as it escapes when a wound is made; (2) The latex of the uninfected cells coagulates quickly, making the entrance into these cells mechanically difficult; (3) The uninfected latex cells collapse because of the pressure of neighboring cells when their own internal pressure is reduced by the escape of latex, thus closing broken parts mechanically.

The writer used a method of inarching, that is grafting by approach, an infected and an uninfected *Asclepias nivea* plant. After the grafts were completed needle punctures were made through the joined surfaces in the hope that infected latex cells of one plant would be broken so near uninfected latex cells of the other that latex of the infected plant would be forced into the latex cells of the uninfected specimens. This method, however, succeeded no better than the others.

Further work is needed, for it may prove possible to devise methods of reducing the pressure of the latex in the uninfected plants within the latex cells, or of reducing the pressure of neighboring cells, or of preventing the coagulation of the latex in the wound, thus facilitating mechanical transfer of latex flagellates from plant to plant.

Other problems. The problems above outlined are those which have been suggested by earlier work. As information is accumulated in this field other points will suggest themselves. Study of insect hosts concerned in the transmission of known flagellates may indicate in which insect groups the carriers of other species may be expected to occur. Study of plant hosts of latex flagellates may lead to generalizations as to which genera and species are capable of becoming infected when in the presence of suitable insects. Some latex plants have never been found infected. This may indicate that their latex is an unfavorable medium for the growth of flagellates. It is not yet known whether flagellates of this group can pass from insect to insect without the intermediate step in latex. The testing of new plants as commercial sources of rubber will furnish large numbers of problems in this field of the study of the herpetomonad flagellates capable of living in the latex of plants.

CHAPTER XXX

SPOROZOA IN GENERAL

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INTRODUCTION

IT is the object of this chapter to outline in brief the class SPOROZOA and to indicate in particular where material can most easily be obtained for study and investigation. Information with respect to coccidiosis in birds and mammals will be found in Chapter XXXI; malaria in general and certain phases of the study of malaria are treated in Chapters XXXIV to XXXIX inclusive and details of the study of MYXOSPORIDIA, and MICROSPORIDIA and are likewise given special treatment in Chapters XXXII and XXXIII.

The SPOROZOA are treated in all text and reference books on protozoölogy. Those that occur in man and certain lower animals are discussed in Hegner and Taliaferro's *Human Protozoology*, pp. 266-377. Wenyon in his *Protozoology* devotes Volume II, pp. 779-1152, to this class and Calkins in his *Biology of the Protozoa* presents a brief morphological and taxonomic discussion of the SPOROZOA on pp. 415-462. The reader is referred to these books for descriptions and classification of this group. It will be found that each author uses a different classification. In this chapter the classification employed is much abbreviated for the sake of simplicity. Two sub-classes and six orders are recognized as follows:

Subclass I. TELOSPORIDIA
    Order 1. GREGARINIDA
    Order 2. COCCIDIA
    Order 3. HÆMOSPORIDIA

Subclass II. NEOSPORIDIA
  Order 1. MYXOSPORIDIA
  Order 2. MICROSPORIDIA
  Order 3. SARCOSPORIDIA

GREGARINES

The gregarines are among the simplest of the SPOROZOA and are easily obtained for study since they are common parasites in the digestive tract and body cavity of insects and earthworms. One of the easiest types to obtain is *Monocystis* which occurs in the seminal vesicles of the earthworm. The life-cycle of *Monocystis* is fairly well known but many points regarding it still remain obscure and the method of transmission of this type of gregarine needs further investigation.

Cephaline gregarines, that is gregarines with an epimerite or "head," are abundant in insects and can be obtained easily from the intestine of grasshoppers, cockroaches or meal worms. There are a number of problems concerning the life-cycle and activities of the cephaline gregarines, for example, their method of locomotion and the significance of the union of sporonts end to end, a condition known as syzygy. A number of very complicated life-cycles have been described among the schizogregarines, a group of gregarines including a period of schizogony in their life-cycle. Certain stages are passed in one host such as a snail and others in another host such as a crab. Confirmation is needed of certain of these life-cycles.

COCCIDIA

COCCIDIA are likewise easy to obtain for study. Perhaps those that occur in rabbits are most prevalent. A large proportion of these animals are infected, and unsegmented oöcysts are usually present in their feces; the segmentation of these can be observed if material is placed in a five per cent aqueous solution of potassium bichromate, which inhibits the growth of bacteria. Other laboratory and domesticated animals such as rats, cats, dogs and chickens are frequently infected with coccidia. In fact it is often difficult to prevent infection with species belonging to this order. The common species that occurs in the rabbit belongs to the genus *Eimeria* in the oöcysts of which there are four spores, in each of which two sporozoites develop. Species belonging to the genus *Isospora* may be obtained from cats and dogs; in their oöcysts two spores are developed each containing four sporozoites. A single coccidium, *Isospora hominis,* is known with certainty to occur in man. It appears to be rare. Very little is known about this species except the development within the oöcyst, hence we must assume the rest of the life-cycle of this species from what we know of the life-cycle of species belonging to the same genus that occur in lower animals. Dobell (1919) named several species of coccidia from man which were later proved by Thomson and Robertson (1926*a,* 1926*b*) to be parasites of fish, the oöcysts of which had been eaten by the human host, had passed through the intestine and been found in the feces. The oöcysts of the coccidia are very resistant and it is thus necessary for one to be certain that he is dealing with an actual parasite of any particular host and not with a foreign parasite that has simply passed through the alimentary canal. Cross-infection experiments, especially by Andrews (1927), have yielded many interesting facts regarding the host-parasite specificity of coccidia. Many problems are open for study involving the host-parasite relations of members of this group. (See Chapter XXXI.)

HÆMOSPORIDIA

Among the HÆMOSPORIDIA are a number of families and genera of great interest. The genus *Plasmodium* which contains the malarial parasites will be treated in general in Chapter XXXIV. Species belonging to other genera that may be obtained without great difficulty are as follows:

The genus *Hæmoproteus* occurs in the endothelial cells of the blood vessels or in the red blood cells of vertebrates. Many species of common birds are infected with *Hæmoproteus.* The best known species is *H. columbæ.* In tropical and semitropical regions this species is transmitted from pigeon to pigeon by a hippoboscid fly of the genus *Lynchia.* How *Hæmoproteus* is transmitted in regions where *Lynchia* does not occur is not known. The life-cycle of *Hæmoproteus* has been described especially by Adie (1915, 1924) but needs further study. Many attempts have been made to transfer infections from one vertebrate host to another by blood inoculations without success. If an easy method of transfer could be devised a number of problems in the host-parasite relations of *Hæmoproteus* could be attacked with profit.

To the genus *Leucocytozoon* belong parasites that occur in the blood cells of birds. Very little is known regarding the life-cycle and method of transmission of the members of this genus. Many species have been named but largely on the basis of host rather than on morphological differences. Hartman (1927) has studied leucocytozoa from birds in this country.

Hæmogregarines are unpigmented parasites that occur in both red and white cells in the peripheral blood of mammals, birds, amphibia, and fish. Species most easily obtained for study belong to the genera *Lankesterella* and *Karyolysus;* these occur in the blood of frogs. An excellent account of a mammalian hæmogregarine is that of Miller (1908) who studied *Hepatozoon muris,* the asexual stages of which occur in rats and the sexual stages in the rat mite. Another hæmogregarine that has been described in detail is *Hæmogregarina stepanowi* (Reichenow, 1910) which occurs in the tortoise and the leech. Hæmogregarines have been described from man but, as Wenyon (1923) has pointed out, these probably were abnormal specimens of other parasites, or artifacts.

The family PIROPLASMIDÆ contains several species that are available for study in certain parts of America. One of these is the parasite of Texas fever in cattle, *Babesia bigemina.* This species, as Smith and Kilbourne (1893) proved, is transmitted by ticks and passes by "heredity" from the mother tick by way of her eggs to the young ticks. The life-cycle of this organism in the tick is unknown. Dogs, monkeys and many other domesticated and wild mammals have been found to be infected with species of *Babesia* to which separate specific names have been given. The species in the dog, *Babesia canis,* is probably better known than that in any other mammal.

Certain genera have been described in the order HÆMOSPORIDIA that are doubtful in nature. *Toxoplasma* has been reported from various species of mammals and birds and even from man. *Cytamœba bacterifera* is an organism that occurs frequently in the red blood cells of frogs in the United States and other countries. Its nature and systematic position are doubtful (Hegner, 1921). Minute spherical bodies that appear near the margin of the red cells of cattle and other animals, both in this country and abroad, have been considered by various investigators to be PROTOZOA and placed in the genus *Anaplasma.* They appear to consist almost entirely of chromatin. They may not even represent living organisms. They resemble what are known as "Jolly bodies" which frequently occur within the red cells of men and other animals.

NEOSPORIDIA

The MYXOSPORIDIA are parasites of cold-blooded animals, principally fish. We owe to Kudo (1919) the most extensive monograph of this group. To Kudo we are indebted also for an extensive monograph on the MICROSPORIDIA most of which occur in arthropods. These two groups need not be considered further in this place.

The SARCOSPORIDIA are muscle parasites of vertebrates. They occur in reptiles, birds and mammals. The best known species is *Sarcocystis muris* that occurs in rats and mice. Other SARCOSPORIDIA are common in sheep, cattle and horses. Very little is known regarding the method of transmission of the SARCOSPORIDIA. Several cases of sarcosporidiosis have been described from human beings. It has been suggested that these were really species that normally occur in other animals and that accidently succeeded in invading the muscle of man.

CHAPTER XXXI

COCCIDIOSIS IN BIRDS AND MAMMALS

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METHODS

Collection and Concentration of Oöcysts. Coccidial oöcysts may be collected either from the feces of an infected animal or from its intestine. If the material is obtained from either source when the animal is at the height of its infection, it is not necessary to undertake any very elaborate measures for concentration. The feces or scrapings may be diluted with from ten to twenty volumes of water, strained through several thicknesses of cheesecloth, and gently centrifuged for two or three minutes. The sediment contains oöcysts in great numbers with a minimum of débris.

If it is necessary to obtain a heavier concentration of oöcysts than the feces of the available infected animals can supply, the stools may be treated as follows: The specimens collected should each be examined by the simple smear method (see section on *Diagnosis*), and samples containing so few oöcysts that it is difficult to find them by this method should be discarded. The stools are diluted with about twenty times their volume of an aqueous solution of one per cent chromic acid or five per cent potassium bichromate. This solution will deodorize the feces and make them much easier to handle. The mixture is thoroughly emulsified by stirring, shaking, or agitating with a mechanical mixer. It is then poured through a filter made of four thicknesses of cheesecloth. The residuum should be stirred and washed with a small quantity of water and the washings added to the filtrate. The filtrate, which will contain most of the oöcysts, is dispensed into graduated 1000 milliliter separatory funnels and is allowed to sediment. After standing at least three hours, the supernatant fluid is decanted through the top of the funnel, the sediment being carefully retained. Water is added to make up the original volume, the funnel is shaken vigorously, and the contents are permitted to sediment as before. If time allows, it is well to continue this washing by sedimentation until the supernatant fluid appears to be perfectly clear after three hours' standing. Two sedimentations will, however, suffice to free the oöcysts of most of the light suspended matter which is present with them. After the final decantation, the original volume is restored with a boiled saturated solution of common salt and is thoroughly shaken. The increased specific gravity of the liquid containing the oöcysts causes the organisms to float. After this solution has stood for an hour, it is drawn off from the outlet at the bottom of the funnel until the layer of floating particles reaches the stopcock. This material is saved in the funnel and is again diluted with water. Enough water must be added so that the retained volume is increased at least three times. The specific gravity of the solution is now reduced below that of the oöcysts so that they will sink to the bottom. They are permitted to sediment overnight, after which the oöcyst-containing layer may be drawn off from the bottom outlet either directly in receptacles for sporulation or into fifty cubic centimeter centrifuge tubes for further concentration.

Sporulation. Investigations upon the coccidia of birds or of mammals usually involves the experimental infection of animals to act as hosts. To successfully infect new animals, it is necessary to have mature oöcysts, that is, oöcysts which contain their final number of viable sporozoites within their sporocysts. Oöcysts passed by animals during the course of the infection are unsegmented. They contain a single contracted (if the individual organism has been fertilized) mass of protoplasm. Under suitable conditions of moisture, oxygen tension, and absence of bacterial putrefaction this protoplasmic mass will fractionate into a number of sporoblasts (specific for the species) and these sporoblasts will again subdivide into a number of sporozoites. The three following methods of sporulating oöcysts have been used by the writer with success:

The usual method of accomplishing this sporulation is to place feces containing immature oöcysts into a solution which will prevent growth of bacteria without injuring the coccidia. A five per cent solution of potassium bichromate is very satisfactory for this purpose. To provide a sufficient exposure to atmospheric oxygen, the mixture of bichromate and feces should be contained in flat-bottomed dishes such as moist chambers of Petri dishes. The layer of the mixture should be not over one centimeter in depth. Fluid lost by evaporation should be replaced daily as desiccation will destroy the oöcysts. The receptacle must *not* be covered as air is necessary for the sporulating oöcysts. Kept at room temperature, mature oöcysts may appear any time after forty-eight hours. The feces containing the oöcysts may be washed to remove dirt, straw, etc., either before or after sporulation. Washing is easily accomplished by mixing the material with from five to ten volumes of water and straining through four thicknesses of cheesecloth. The original volume of feces may be approximately restored by centrifugation or sedimentation.

A second method of sporulation is as follows: large Petri dishes or moist chambers are prepared by lining the bottom with at least six layers of filter paper which has been saturated with a two per cent solution of formalin. Fresh, unwashed feces are placed upon the wet filter paper. The formalin vapor prevents the interference of bacterial putrefaction, and the fact that the material is not submerged in fluid facilitates the ventilation of the mass and thus promotes sporulation. The contents should be sprinkled daily with water or with a one per cent formalin solution. The container must *not* be covered. If the oöcysts are kept at room temperature, they will commence to segment within forty-eight hours. After sporulation is complete, the feces may be washed off the filter paper, strained, and concentrated. It is important to remember that oöcysts prepared by this method for the infection of animals must be *thoroughly* washed to remove all traces of formalin which might injure the animal receiving them.

Either of the above methods will work faster at incubator temperature. The difficulty is that unless carefully watched, the material will dry out due to the rapid evaporation occasioned by the higher temperature. The following method has been designed to circumvent this problem, and has frequently yielded mature oöcysts after incubation over night. Feces containing oöcysts are strained, sedimented, mixed with five per cent potassium bichromate and placed in large Petri dishes in a layer not more than a half centimeter in depth (Fig. 18, C). A 250 cubic centimeter aspirator bottle (Fig. 18, A) with an outlet near the bottom is provided with a burette tip and glass stopcock (Fig. 18, B) on a curved stem set into a rubber stopper, as indicated in Fig. 18. The bottle is filled with water and is placed on a shelf in the incubator so that the contents of the bottle can drop into the Petri dish. The stopcock is so regulated that the tip delivers a drop of water every two or three minutes. This compensates amply for the evaporation of the oöcyst-containing solution, and sporulation goes on rapidly. The use of this method has led to the quickest yield of the largest proportion of ripe oöcysts of any method used in this laboratory.

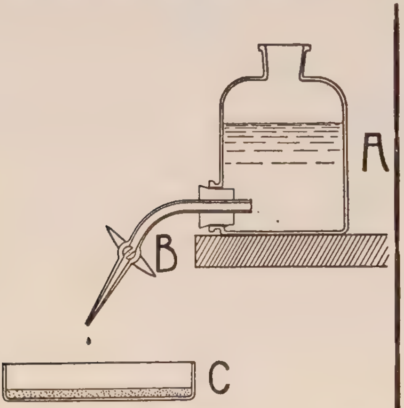

Fig. 18.—Apparatus for maintaining moisture content of segmenting oöcysts at incubator temperature. For explanation see text.

Inoculation. "Inoculation" is used here rather loosely to indicate the introduction of organisms capable of starting an infection into the body of a susceptible host.

Coccidia may be inoculated into animals by three general methods, namely, (1) feeding, (2) injection into the stomach, and (3) by surgical manipulation.

The first method is comparatively simple and suffices when it is required to inoculate an animal without regard to the exact amount or number of organisms introduced, or when it is desirable to avoid the use of an anæsthetic. The animal to be inoculated should be deprived of food and drink for at least twenty-four hours before the attempted inoculation. The oöcysts should be washed free of any irritating or unpalatable chemical with which they may have been in contact, and they should be concentrated by centrifugation into the smallest volume which is convenient to handle. Rats and mice may be easily infected by placing them in a cage with a watchglass containing the concentrated oöcysts in two or three drops of milk. Cats may be inoculated in a similar manner by using a slightly larger quantity of milk. It is not always easy to persuade rabbits and guinea-pigs to drink milk, but if the oöcyst mixture is spread on the split surface of a carrot it will be quickly ingested. Dogs will take the inoculum readily if it is injected with a hypodermic syringe into a frankfurter. The concentrated oöcysts may be placed into a small capsule (No. 3 or 4) and then administered to a cat, dog, or rabbit. In this way, a measured quantity of oöcysts can be given to the animal, but this method lacks what may be an essential condition of the experiment, namely, mixture with salivary secretions.

Animals are inoculated with coccidia most easily by injecting the inoculum directly into the stomach. A stomach tube for small animals such as rats, guinea-pigs, rabbits, chicks, kittens, and puppies is readily contrived by cutting off the large open dilated end of soft rubber catheters (No. 5 small to No. 15 large, according to the size of the animal) and slipping it over the end of a Luer syringe. Hard silk web catheters may be similarly used, and they are undoubtedly easier to manipulate due to their greater rigidity. This same advantageous feature, however, is frequently responsible for perforation of the esophagus, so that the softer catheters are preferred. With larger cats or dogs, an infant's size stomach tube with funnel is more useful than the catheters.

The passage of a stomach tube is greatly facilitated by stretching the head back as far as possible to reduce the pharyngeal angle. This is particularly essential in the rodents as the proximal position of their molars causes interference. It is well to lubricate the tube by dipping the end of it into paraffin oil. The tube should be held against the dorsal surface of the pharynx and firmly pushed down the esophagus. It must be pushed by the cardiac sphincter at which point a slight resistance will be felt, otherwise back flow may develop and fluid will get through the glottis and down the animal immediately. Once the tube is in position, a measured quantity of fluid containing a known number of oöcysts may be administered. If the large stomach tube and funnel is used, it is well to pour a small quantity of water through the tube to wash through organisms which might be adherent to the walls.

It may or may not be necessary to anesthetize the animal before passing the stomach tube. Dogs of any size, unless they are vicious, will tolerate the operation without anesthesia. Cats should be stupefied with ether before attempting intubation, though it is not necessary with kittens. Rabbits as a rule do not require an anesthetic, but guinea-pigs and rats should be etherized. Chicks require no anesthesia.

The third method of inoculation permits the introduction of infective stages belonging to the asexual cycle, merozoites, as well as mature oöcysts. Merozoites are collected by scraping infected portions of the intestine of a freshly killed host. The scrapings are immediately suspended in warm (37° C.) Ringer's fluid, strained through several thicknesses of cheesecloth to remove débris which would clog the needle and are then aspirated into a warm Luer syringe provided with a twenty gauge needle. The syringe and contents are kept at incubator temperature until use. Just before use, a drop of the contents is expressed upon a clean slide and examined under the microscope to be sure that the merozoites are present and viable (as evidenced by their motility). Briefly, the procedure is (1) to prepare the inoculum (a suspension of either merozoites or oöcysts), (2) anesthetize the recipient, (3) prepare it for abdominal section, (4) expose the site of injection (stomach, duodenum, jejunum, ileum, liver, etc.), (5) inject inoculum, and (6) repair the abdominal incision.

It is many times necessary to perform inoculations quantitatively. This may be done by washing and concentrating a quantity of sporulated oöcysts. The suspension is *thoroughly* shaken, and a drop is placed in the chamber of an ordinary hemocytometer. Only the sporulated oöcysts from the entire ruled space (nine square millimeters) are counted, and the number on this area is divided by 0.9 which will give the number per cubic millimeter. An average of at least ten counts should be taken. The suspension may then be adjusted by dilution to a content of oöcysts which is convenient for computation, *e.g.,* 10,000 oöcysts per cubic centimeter. This stock solution should be protected against growth of bacteria or free-living protozoa by the addition of a drop or two of strong formalin. The number of oöcysts should be counted at various intervals as a few of the unsegmented oöcysts not originally included in the count do go on to development, thereby increasing the number, and there is a constant low rate of mortality among the mature oöcysts which tends to decrease the number. Coccidial oöcysts are heavier than water and quickly fall to the bottom of any solution whose specific gravity is 1.00 or less. Consequently it is necessary to shake a counted suspension before withdrawing a sample, and if a syringe is used in injecting them, the contents must be evacuated while the syringe is in an upright position, *i.e.*, plunger on top, outlet at bottom.

Other methods of estimating or of counting the number of oöcysts in a suspension have been used. Johnson (1927) "estimates" the number of oöcysts present by direct microscopic examination of a very small drop (0.02 of a cubic centimeter) on a slide. In a later piece of work (1927*a*), his method is to count the number of sporulated oöcysts in 0.01 of a cubic centimeter of a dilution of the original suspension.

It must be realized that counts of these organisms unless made on very large samples immediately before use cannot be very accurate. Under the very best conditions it is probably not possible to exclude all non-viable oöcysts from the count. Furthermore it is possible that unsegmented oöcysts which were not included in the count may mature during their passage through the host. Nevertheless, these irregularities must tend toward consistency from one experiment to another. It is the writer's belief that this quantitative technique with all its difficulties is of value, in that it permits one observer to verify and extend the results of another under approximately the same conditions.

Diagnosis. There are a number of methods of diagnosing coccidial infections, all of them based on the finding of oöcysts. They may be roughly divided into methods in which the material examined is concentrated, and the single method in which it is not.

The simple smear method is the quickest of all methods, and, except in heavy infections, it is the least accurate. An amount of stool is gathered on the end of a toothpick and is rubbed up in a drop of saline or water on a slide. A coverslip is dropped on this suspension. The completed preparation should be just thin enough to permit the reading of newspaper print through it. The entire area under the coverslip is examined, and in the event of a negative finding, one or two more similar preparations should be examined.

All other methods of diagnosis depend upon a concentration of the material examined. Concentration is accomplished either by centrifugation, by an increase in the specific gravity of the material containing oöcysts, or by a combination of centrifugation and flotation.

Davis and Reich (1924) mix the fecal specimens with water and allow them to soften if they are hard. They are then washed and strained through gauze to remove the coarser particles. The filtrate is centrifuged at a speed of between 1200 and 1500 revolutions per minute for ten to fifteen seconds. The supernatant fluid is poured off and the sediment is washed and re-whirled at the same rate from one to three times, depending upon the amount of fine, light material to be eliminated. The final sediment may be mixed with water, spread upon a slide, and examined without using a coverslip.

Nöller and Otten (1921) employ flotation with a salt solution. A portion of stool about the size of a pea is mixed with saturated salt solution, adding the saline drop by drop until a homogeneous mass is obtained. This is poured on a strainer (0.2 of a centimeter mesh) and is forced through by pouring on saturated saline and at the same time stirring the mixture. The filtrate is poured into Erlenmeyer flasks of capacities varying from 25 to 200 cubic centimeters according to the quantity of stool available. The fluid should come up into the neck of the flask. The proportion of feces to salt solution varies from one to eight to one to twenty, but may be as high as one to 400 without materially affecting the result. The flask with its contents is allowed to stand for from twenty-five to ninety minutes. Then a portion of the surface is removed with a platinum loop 0.3 to 0.4 of a centimeter in diameter and is examined on a slide under the microscope.

Vajda (1922) has described a method for the detection of helminth ova that can be used equally well for coccidia. He uses glycerin to increase the specific gravity of the oöcyst-containing fluid. A watery suspension of feces is treated with from one to three parts of glycerin (sp. gr. 1.25) and is well mixed. The coccidia rise to the surface from which they may be removed with a glass rod. Refinements of this method call for a straining of the feces-glycerin mixture through linen cloth, centrifugation at a moderate speed for from fifteen to twenty minutes, and a mixing of the sediment with from one and one-half to three parts of glycerin. The author suggests removal of the floating organisms for examination as follows: Fasten filter paper by means of a string at the mouth of a test-tube and dip it into the feces-glycerin mixture until the whole surface of the filter paper touches the surface of the liquid. This method of diagnosis can be easily adapted for purposes of concentrating large quantities of oöcysts.

Sheather (1923) has devised a method combining sugar flotation with centrifugation. The various steps in his technique as summarized in his paper are as follows:

"1. Emulsify the feces in water until their mobility approaches that of water.

"2. Strain through wire gauze having thirty meshes to the linear inch.

"3. Mix the filtrate with an equal volume of a solution of sugar in water prepared by dissolving one pound in three-quarters of a pint (sp. gr. 1200).

"4. Centrifuge for two minutes at 2000 to 2500 revolutions per minute.

"5. Lift the eggs from the surface of the liquid by lowering a cover-glass into contact with it, which is simply achieved by attaching the cover-glass to a handle by means of a small 'beak' of plasticine, and on withdrawal detaching this on the surface of a slide."

The direct centrifugal flotation method used by Lane (1922) for the mass diagnosis of hookworm infestation has been modified (Andrews, 1926) for use in the diagnosis of coccidiosis.

"Strong Erlenmeyer flasks of sixty-five or seventy cubic centimeters' capacity are calibrated and etched to indicate volumes of fifty-six and fifty-eight cubic centimeters. The flask is filled with water up to the level of the fifty-six cubic centimeter mark, and then a sufficient amount of the specimen stool collected at random from various parts of the feces is added to bring the fluid level up to the fifty-eight cubic centimeter mark. Thus two cubic centimeters of stool have been added. Four or five cubic centimeters of lead shot (medium size and small, mixed) are poured into the mixture. The flask is closed with a rubber stopper and is shaken until the contents are uniformly mixed. If the stool is hard, it is futile to try to comminute it by shaking with shot unless it has stood in water for several hours. The most convenient routine is to let the stoppered flasks stand with their contents overnight and to shake them the following morning. The thoroughly emulsified specimen is then poured into a round-bottomed fifty cubic centimeter centrifuge tube which has been calibrated and etched to indicate a volume of forty-five cubic centimeters. The centrifuge tubes are balanced in their cups by the *addition* of water and are whirled at the lowest speed of the centrifuge (International Electric Centrifuge, Size 1) for two or three minutes.

This manipulation sends all of the oöcysts to the bottom of the tube. The supernatant fluid is carefully decanted, all of the sediment being retained. A boiled saturated solution of sodium chloride is then added in small amounts, while the tube is gently shaken so that the sediment is loosened and mixed with the saline. About forty-five cubic centimeters of the solution are added in all. The tubes are balanced by the addition of the saturated saline and are centrifuged, at the same rate of speed as before, for not longer than a minute. A portion of the upper layer of the mixture is lifted off, by suspension across the mouth of a small vial, on to a glass slide and is quickly examined under the microscope. If oöcysts are present, they will be found floating in the surface layer.

"This method is advantageous in that a comparatively large sample (0.77 of 2 cubic centimeters, or approximately 1.5 cubic centimeters) is examined. Inasmuch as the sample is a composite one, it is clearly much more representative of the total stool than the sample used for simple smear examination. Comparative estimates of the intensity of the infection, as judged by the numbers of discharged oöcysts, are possible because the samples are volumetrically equivalent. Just how much more efficient the D. C. F. method is than the simple smear method has not been determined,[1] but on several occasions, oöcysts were readily demonstrated by the former method when long searching of smears from the same material did not reveal any."

Enumerative Technique. The coccidial oöcysts of most animals are of sufficient size and resistance so that various methods devised for the purpose of counting helminth ova may be applied with only minor adaptations to the enumeration of coccidial oöcysts. Such techniques are very valuable in following the course of experimental infections in actual quantitative terms of parasite production, or in comparing various infections in terms of oöcysts discharged.

The Stoll dilution egg-counting method has been used for counting coccidial oöcysts from the cat and dog with very satisfactory results. The original papers (Stoll, 1923, Stoll and Hausheer, 1926,

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[1] Hausheer and Herrick (1926) have shown that 500 hookworm eggs per gram of stool must be present to insure a positive diagnosis by the smear method, whereas as few as 20 per gram are always detected by the D. C. F. method.

1926*a*) contain details concerning technique and accuracy. A synopsis of the method is given below (Stoll and Hausheer, 1926, p. 82).

"For dilution egg counting one or more grams of stool, weighed by difference, are put into a tube marked so that decinormal sodium hydroxide may be added to secure a ratio of one gram of feces to fifteen cubic centimeters total mixture with the diluent. Usually three grams are weighed into a tube marked at forty-five cubic centimeters; five grams raised to seventy-five cubic centimeters have also been recommended and employed for field use, the larger amount helping to reduce any percentage weighing error in routine. After adding the diluent, glass beads are introduced to assist in comminution, the tube is rubber-stoppered, and shaken thoroughly, to secure, first, a complete setting free of the ova from the fecal constituents, second, a homogeneous suspension of the eggs in the mixture. By means of an accurately calibrated pipette 150 cubic millimeters (.15 of a cubic centimeter) of the mixture is immediately transferred to a slide, and the ova counted with the aid of a coverslip of appropriate size to cover the drop. Originally 22 x 40 millimeter covers were suggested and have been used in this study; 24 x 40 millimeter covers have also been used in routine; 24 x 50 and 35 x 50 have occasionally been employed. A mechanical stage and the low power of the microscope (usually 10x ocular and 16 millimeter objective with drawtube length of 160 millimeters, producing an enlargement of 100 diameters) were used in making the counts. According to the dilution and drop size the number of ova on the slide represents theoretically 1/100th the number per gram of original feces; in practice not less than two slides are counted to secure an average result, as is usual in dilution methods."

An alternative technique is a modification of the direct centrifugal flotation method devised by Lane. (See papers published 1922, 1924, 1924*a*, and 1925.) As used by the writer for cat and dog coccidia, the steps in the procedure are as follows: two grams of stool (three grams may be used if the infection is very light, or one gram or less may be used if the number of oöcysts is so high that it is difficult to count them from a two gram suspension) is weighed into a dry Erlenmeyer flask which is calibrated at ninety cubic centimeters on the neck. Formed or soft stools are easily handled with tongue depressor blades and six-inch applicator sticks. Diarrheic stools may be handled with either wooden or cardboard "ice-cream spoons." The advantage of these instruments over the metal spatula or spoon is that they do not have to be cleaned and dried for each specimen but can be discarded with each stool, and a fresh set known to be clean, dry, and uncontaminated may be used for the next. The flask is filled with water to the calibration mark, and two or three cubic centimeters of mixed medium and small lead shot are added. The flask is shaken until the contents are uniformly dispersed, and *immediately* after shaking (before the oöcysts have time to settle out), a sample is poured into a "Lane tube" (to be described) up to the fifteen cubic centimeter calibration mark. The tube is placed within the special centrifuge cup (to be described) which is placed in either a special Lane centrifuge or in an ordinary hand centrifuge and is whirled at a speed of 1000 revolutions per minute for two minutes. The Lane tube is withdrawn and the contents (known as the "pour-off") are carefully decanted into another Lane tube, care being taken to retain all of the sediment within the original tube. A boiled saturated solution of common salt is added, a little at a time, shaking the tube gently between additions so that the sediment will be suspended without bubble formation, until the tube is nearly filled. It is placed in the special cup, and saturated saline is added with a pipette until the surface level of the solution is even with the ground edge of the tube. A thick coverglass (18 x 18 millimeters, No. 3) is laid upon the mouth of the tube between the four horns of the cup so that the horns are opposite the middle of the sides of the coverslip. A small piece of paper (about fifteen millimeters square) is dropped on to the coverslip. The entire centrifuge cup and contents is lifted from the centrifuge, and, with the forefinger placed against the paper on the coverslip, is inverted several times, being careful not to spill any of the contents. The cup and tube are replaced in the centrifuge, and are rotated for one minute at 1000 revolutions per minute. The coverslip is removed by firmly grasping it at the corners with the thumb and forefinger and suddenly raising it. It is then mounted on a slide, wet side down, and the oöcysts are counted immediately. The tube is again filled with saturated saline, covered with a fresh coverslip and is manipulated as before. The procedure is "pushed to finality," *i.e.,* coverslip preparations are made until two consecutive preparations are negative. For very accurate determinations, the original "pour-off" is centrifuged, decanted, and whirled with salt solution just as the original sample until two negative coverslips are demonstrated. The sum of the oöcysts counted in sample and pour-off is multiplied by three (oöcysts were counted from ${}^{15}/_{90}$ths of two grams) the result being expressed as "oöcysts per gram." If the specimen was obtained from a thoroughly mixed twenty-four hour sample, the figure obtained may be multiplied by the weight of the stool and expressed as "oöcysts per day."

All of the apparatus for this technique, centrifuge, cups, tubes, and coverslips, designed according to Lane's specifications, may be obtained from R. B. Turner and Company of London. A satisfactory assembly may be obtained in this country at no greater expense and at a considerable saving of time.

The ninety cubic centimeter Erlenmeyer flask may be readily made by invaginating the bottom of a 100 cubic centimeter Pyrex flask. This is done by providing the flask with a one-hole rubber stopper. A short glass tube (three or four centimeters long) penetrates the rubber stopper and is attached to a rubber tube of a convenient length that will reach the mouth. Heat is applied to the bottom of the flask, first as a carbonizing flame and later as a blast. When the bottom begins to soften and appear red the flask is removed from the flame, and a negative pressure is created within by suction on the rubber tube. The bottom is raised about one centimeter (see Fig. 19, C). The flask is then annealed and cooled. It is calibrated either with water or mercury. The ninety cubic centimeter mark should come just to the base of the neck as indicated in Figure 19, C. If it comes below or above, the flask must be reheated and

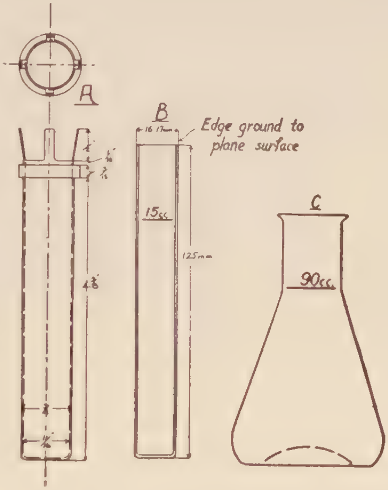

Fig. 19.—Special apparatus for use in modified Lane direct centrifugal flotation method of counting oöcysts. For explanation see text.

the bottom either further invaginated or blown out to adjust the volume.

A Lane tube (Fig. 19, B) is a cylindrical glass tube, closed and flattened at one end. Its length overall is 125 millimeters and its outside diameter is sixteen to seventeen millimeters. The upper edge is ground perfectly plane, and may be cautiously heated in a flame until the glass is just beginning to soften. The plane surface must not be destroyed by the heating. This softening of the edge prolongs the life and usefulness of the tube by preventing the chipping at the top which readily occurs otherwise. The tube is calibrated and etched to indicate fifteen cubic centimeters.

The special centrifuge cup (Fig. 19, A) is best constructed from a single piece of brass rod, bored and turned as illustrated. This cup may be used with a standard four-place head on a hand centrifuge. A heavy plating of nickel is a partial protection against the corrosion from the salt water.

Of the two methods of counting oöcysts, Stoll's dilution method is probably to be preferred, as it requires less apparatus to carry out the procedure, and much less time to complete the determination. According to Hausheer, Herrick and Pearse (1926) the time required to complete a Lane determination is from 109 to 125 minutes, whereas similar samples counted by the dilution method (two slides counted) required from thirty to forty minutes. On the other hand, the Lane method counts *all* of the oöcysts in a measured quantity of stool and therefore is particularly useful in picking up light infections and in counting the first few and last few oöcysts of an experimental infection. When the infection is heavy, it is almost futile to try to use the Lane method as the concentration of oöcysts on the first few slides is so high. This difficulty may be met by dilution of the sample, but in so doing the advantage of counting the entire number of eggs is lost.

In any method of counting oöcysts it is necessary, as with helminth ova, not to lose sight of the fact that the concentration of oöcysts must vary with the water content of the stool. Thus mushy, or unformed stools contain more water and therefore a smaller number of oöcysts per unit volume or weight than feces which are formed, but which are otherwise equivalent. Similarly, a diarrheic stool will be diluted, as regards oöcyst content, even more than a mushy one. Just what the ratio of dilution is under these various conditions will probably have to be worked out for each type of animal used. Stoll (1923*a*) found, dealing with hookworm ova in human feces, that it was necessary to adjust counts made on mushy or diarrheic stools to the basis of formed feces by multiplying the egg-count on mushy stools by two, and on diarrheic stools by four. Herrick (1928) found that the variability in counts of hookworm eggs in canine feces was fifty-one per cent greater when the feces were classified as formed, mushy, and diarrheic and adjustments are made on the basis of the 1 : 2 : 4 ratio than when they are counted without regard to consistency.

Excystation. It is possible to produce excystation of coccidial oöcysts *in vitro* or *in vivo*. The process has not been exhaustively studied. The technique involved is useful not only as a means of inquiring into the nature of the process *per se* but should be a valuable adjunct in the study of the mechanism of host-parasite specificity, as a means of determining the viability of oöcysts, and will perhaps assist in other similar procedures.

In vitro excystation has been best described by Krijgsman (1926). His paper contains a fairly complete review of the literature on the subject. He uses the washed concentrated oöcysts from the feces of the rabbit. After these have been centrifuged in distilled water, the water is replaced by a solution of pepsin in 0.5 per cent hydrochloric acid. They are exposed to the action of this enzyme for thirty minutes, after which they are washed by centrifuging in two changes of distilled water. The sediment containing the oöcysts is suspended in a hanging drop of trypsin in slightly alkaline solution. Excystation commences within two hours. Preliminary experiments showed that while excystation may be produced by simple immersion in the pancreatic enzyme, the time required is eight hours or over. Exposure to gastric enzymes did not result in excystation but produced certain physical changes in the cyst wall which were undoubtedly the effect of the acid. Thus excystation of coccidial oöcysts, according to Krijgsman is a process analogous to protein digestion—with gastric enzymes playing a predigesting rôle, and pancreatic enzymes completing the liberation of the sporozoites.

Excystation *in vivo* has been repeatedly carried out by the author, using the following simple technique (Andrews, 1930). Apparently any species of coccidia in birds and mammals can be used. Coccidia from cat, dog, guinea-pig, rabbit, pig, and prairie-dog have excysted with equal success. Young rats (75 to 100 grams) are deprived of food and water for twenty-four hours prior to the experiment. This has the double advantage of making the animal eager to eat, and of thoroughly emptying the stomach and small intestine. Oöcysts are washed by centrifuging in water. It is necessary that chemicals which may have been used to prevent putrefaction be entirely removed. The supernatant water is poured off and the oöcysts are recentrifuged in a small quantity of sweet milk. The supernatant milk may be discarded and the oöcysts remain concentrated in a few drops of milk. This material is offered to the starved rat and is immediately consumed. Sixty minutes after the ingestion of the oöcysts, the rat is killed and the intestine is removed. At various points segments of the small intestine will appear to be dilated and to have white contents. The intestine should be opened at these points and smears made of the contents. Various stages of excystation will be readily found. A small quantity of fine powdered charcoal may be added to the milk-oöcyst mixture before it is administered, if it is desired to identify the experimental food and to make a precise determination of the distance the food has traveled in the intestine. This technique has been used by Chapman (1929) in an attempt to obtain pure coccidia protein for immunological purposes.

PROBLEMS

Treatment. Undoubtedly the most important problem relating to coccidiosis is the treatment of it, particularly in domestic animals. As far as is known, coccidiosis appears to be a very rare disease of man, some two hundred cases having been reported to date. It is possible, however, due to ignorance of the clinical manifestations that many cases have been missed. Reasoning by analogy from the behavior of the acute infections in young animals it might be expected that the most severe symptoms and that death, if the infection is ever fatal to man, would occur before the sexual phase is well under way. Inasmuch as the present methods of diagnosis are all based upon the recovery of the oöcysts it is easy to see that severe cases might go undiagnosed because of the absence of oöcysts in the feces at the time of examination, and the mild cases, not requiring an examination of feces, might likewise go unrecognized. Because of the paucity of cases in man, few therapeutic measures have been attempted, and none have been established as a specific treatment.

Among the coccidial infections of lower animals, bovine coccidiosis is probably the most important economically, with poultry coccidiosis coming next. Tremendous losess occur each year, due to coccidiosis of fur-bearing animals, particularly rabbits and foxes raised on a commercial scale. Coccidiosis of swine, sheep, goats, pets and experimental animals adds to the importance of discovering some effective method of treating the disease. Animals have been treated with a vast variety of drugs, particularly the compounds and dyes known to possess more or less parasiticidal activity, but without any degree of success that other investigators have been able to confirm. A certain amount of work has also been done on the relation of diets to coccidiosis. Perhaps the most outstanding of the recent contributions on this subject is the work of Beach and his collaborators (Beach and C'Orl, 1925; Beach, 1925, and Beach and Davis, 1925) on the coccidiosis of chickens. Using percentage mortality as a criterion of the efficacy of their treatment, these workers found that a diet containing forty per cent skim-milk powder was very effective in controlling experimental poultry coccidiosis. They attributed the protective property of this diet to its high content of lactose (50.6 per cent) which, they showed experimentally, lowered the pH of the cecal contents from a normal range of 6.0 to 7.4 to a range of 4.4 to 5.6. It was believed that the increased hydrogen-ion concentration of the cæcal contents was injurious to the merozoites. This treatment did not eliminate coccidiosis but considerably reduced the percentage mortality. Further investigations of this type are needed.

Host-Parasite Specificity. The problem of host-parasite specificity probably ranks next to that of therapy in coccidiosis. Man is concerned to know whether or not the coccidia of the lower animals are ever infective to him, whether or not the rats which live in the barn are susceptible to infection by rabbit coccidia and whether or not they can transmit it to cattle. Are the English sparrows which fly from one poultry yard to another capable of transmitting poultry coccidiosis biologically? A considerable quantity of evidence has accumulated (see Andrews, 1927, for literature on host-parasite specificity of coccidia of mammals), most of which tends to establish the fact that the coccidia of one species of host are not infective to another. One notable exception to this rule appears to be the cross-infectivity of the coccidia of cats and dogs. More accurate information as to the extent of this host-parasite specificity is needed. Cross-infectivity seems to be an easily testable experiment to make, yet the results may be very misleading unless one bears in mind three things: (1) the natural coccidial parasites of the host, (2) the interference of an acquired immunity, and (3) mechanical carriage. The first difficulty amounts to very little if the foreign parasites employed differ decidedly in their morphology from the coccidia which are natural parasites of the experimental host. The interference of immunity by virtue of a previous infection is a phenomenon the significance of which is hard to estimate at present. Varying degrees of resistance to subsequent attempts at coccidial infection have been indicated in cat, dog, and poultry coccidiosis. The length of time that coccidial immunity lasts is not known, nor is it known whether or not infection by one species can confer resistance to infection by related species. It is clear, then, that there is a possibility that a negative cross-infectivity experiment might indicate *individual* immunity instead of *species* immunity. In regard to the third caution, mechanical carriage, it is known that non-viable and unsegmented oöcysts will pass through the intestine unchanged. The recovery of such oöcysts must not be mistaken for evidence of the establishment of an infection. Proof of the ability of one species of coccidium to parasitize more than one species of host involves the use of foreign hosts that are free and that are known to have been free from coccidiosis from birth, and the demonstration not only of the discharge of oöcysts from these foreign hosts but of the presence of the asexual forms of the parasite in the tissue of the host as well.

The mechanism of host-parasite specificity offers many interesting problems for investigation. At what point in the development of the "infection" is the foreign host protected? Is there any selectivity involved in excystation or, granting that sporozoites are liberated, are they incapacitated before they can penetrate cells? Are there such differences in the epithelial cells of various animals that the sporozoites of a particular species of coccidium can penetrate into the cells of a particular species of host and be unable to enter the cells of other species of hosts? Or is the infection aborted after the intestinal cells have been entered? The rare incidence of coccidiosis of man has been explained upon the assumption that he was an accidental host to the coccidial parasite of some animal with which he occasionally came in contact. This needs experimental support for acceptance.

Immunity. The problem of individual immunity to coccidial infections is closely bound up with the problem of host-parasite specificity as is indicated in the previous section. It seems very probable that a rational explanation of the mechanism of the one phenomenon will help a great deal in understanding the mechanism of the other.

Conflicting reports have appeared regarding the acquisition of immunity from coccidial infections in general. The author (1926*a*) failed consistently in attempts to reinfect cats and dogs which had recovered from an attack of coccidiosis. Nevertheless, cats and dogs which are kept in the laboratory for a long time (a year or more) have shown evidence of repeated attacks of coccidiosis with intervals of apparent freedom from the disease. Whether these repeated infections are newly acquired each time, or whether they are the relapses of a latent infection, as in the case of bird malaria, is not known. Richenow (1921) and Wetzel (1925) state that no immunity is produced in rabbits and that constant reinfection is responsible for the appearance of a chronic infection. Beach and C'Orl (1925) and Johnson (1927*a*) judging from clinical signs believe that chicks become resistant to coccidial infection as the result of previous infections. Young (1929) counting the oöcysts discharged showed that from a parasitological point of view, subsequent infections, experimentally produced, were of equal or even greater magnitude than the first. In working with rabbit coccidia, Chapman (1929) has attempted to demonstrate the presence of circulating antibodies with complement fixation, precipitin, agglutination, lytic, and skin tests. Skin tests were promising, complement fixation was irregular in its results, and the rest were negative. The greatest difficulty in the work was the production of a pure coccidia antigen.

The foregoing indicates some of the many possibilities in the field of coccidial immunity. The following problems are still to be solved conclusively, and will lead to many other questions for investigation:

1. Do coccidial infections produce in their hosts any demonstrable resistance to subsequent infections—
    a. Clinically—are the percentage mortality and the symptoms of the disease reduced in the latter attacks?

b. Parasitologically—is there any reduction in the number of parasites present or in the duration of the infection in the latter attacks?

c. Immunologically—is it possible to demonstrate the presence of circulating antibodies?

2. How long does this resistance last?

3. Does the resistance increase with the age of the host which has not been infected?

4. What is the mechanism of the immunity? (See section on *Host-Parasite Specificity.*)

5. Is it possible to protect animals from severe, naturally acquired infections by quantitative inoculation to produce a mild infection under carefully controlled conditions?

6. Just what are the factors which result in the production of an acute attack or a chronic infection or a change from the one to the other?

Pathogenicity. While it is known that among the lower animals coccidia may appear as pathogenic, or even lethal, parasites it is clear that in the great majority of cases the host survives the infection with little or no apparent injury. Data regarding pathogenicity of coccidia in man are few, but some of the contributions to the subject are of high quality. The situation in man seems to be a reflection of the situation in animals, namely, that coccidia may upon occasion be severely pathogenic. One of the first questions for investigation is a determination of the factors which predispose toward pathogenicity, and should include a consideration of the following variables on the part of individuals: age, sex, previous experience with the disease, faulty nutrition, concomitant infections, derivation of infection from acute or chronic strain of disease, etc.

The mechanism of pathogenicity should be investigated. Does the parasite kill or injure the host with a toxin? Does it affect carbohydrate metabolism? Does it interfere by means of destruction of intestinal epithelium with the proper absorption of food? Is there sufficient destruction of blood elements to provoke the symptoms observed? Are the chemical and physical equilibria of the blood disturbed in the course of the infection? Some work on determinations of the blood changes have been reported by Leger (1926) in man, Yakimoff, *et al.* (1926, 1926*a*) in cattle and white rats, and Sanders (1928) in kittens.

Cause of Sporogony. The life cycle of COCCIDIA has been common knowledge ever since Schaudinn (1900) first showed how *Eimeria schubergi* developed in the myriapod, *Lithobius,* yet the causal sequence between two of the phases has never been elucidated. That the asexual cycle must start with the animal's infection and must continue as long as the animal remains infected seems almost beyond argument, but some time during schizogony the production of sexual forms leading to sporogony must start. Many speculations have been hazarded as to the nature of the factors which bring this about, but none of them are convincing. It is believed by some that after a certain number of asexual generations gametocytes are produced. Some think that age of the host is responsible, oöcysts appearing earlier in older animals. Others believe that it is a question of immunity, successful antibody production of the host resulting in a chronic type of infection with reduction in asexual multiplication and an increase in oöcyst discharge. These hypotheses should be subjected to experimental test.

Most work on coccidiosis uses as a criterion of infection the presence of oöcysts in the stools, and workers are prone to judge the intensity of the infection by the relative numbers of oöcysts dejected. The truth is that the intensity of the infection, that is, the damage done to the host, depends for the most part upon the asexual activity of the parasites, and to a less degree upon the sexual phase, of which the oöcysts are the final product. It is quite conceivable that the asexual cycle may proceed for a time without the production of any oöcysts, after which, due to the operation of the proper factor or factors which result in the discharge of oöcysts, they may appear in the feces. An investigator following such a course of events in terms of oöcyst production might very properly be led to believe that the second appearance of oöcysts was due to reinfection. If there is any quantitative relation between the numbers of oöcysts and schizonts it should be demonstrated if possible. A careful study of the course of coccidial infections from a clinical point of view correlated with adequate counts of oöcysts discharged per day might lead to further understanding of this relation.

Specificity. The differentiation of species of coccidia particularly in those cases where there is more than one alleged species in the host presents problems of prime importance. In most instances the fundamental character of distinction is the size of the oöcyst. Not all protozoölogists are agreed that differences in size alone warrant the naming of new species. Whether or not one size of oöcysts always breeds true has not been worked out in any animal except the chick.

The problem of determining the specificity of the multiple size races is open to at least two methods of attack: (1) the production of clones following inoculations with a single oöcyst, and (2) the correlation of size with other characteristics which pertain to the race in question.

Tyzzer (1925) working on the coccidia of gallinaceous birds has devised methods for isolating and differentiating species which might be readily applied to the coccidia of other animals. With regard to differentiating species, the following is quoted (Tyzzer, *loc. cit.*):

"In the recognition of species it appears to be a safer course to compare various features than to depend on one alone. The characteristics that may be taken into account are the following:

1. The length of the period of development up to the discharge of oöcysts.[2]
2. The time required for sporulation at a given temperature.
3. Host specificity or degree of restriction to a given host.
4. Characteristic habitat or distribution of the organism in its host.
5. Cross-immunity tests.
6. Pathogenicity as determined by both experimental and natural infection.
7. Morphological studies, applying as well to the forms developing in the tissue and not confined solely to the size and shape of the oöcyst.
8. The relation of parasite to host-cell, together with the reaction of the latter."

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[2] This may be referred to as the "prepatent period." See Andrews (1926, p. 787.)

CHAPTER XXXII

MYXOSPORIDIA

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INTRODUCTION

UNDER the former and still most widely followed system of classification of the PROTOZOA which was largely formulated by Bütschli, MYXOSPORIDIA (Bütschli, 1881) was placed in the SPOROZOA (Leuckart, 1879). Schaudinn (1900) divided SPOROZOA into TELOSPORIDIA and NEOSPORIDIA, and placed MYXOSPORIDIA together with MICROSPORIDIA (see next chapter) in the latter group. Doflein (1901) coined the term CNIDOSPORIDIA for MYXOSPORIDIA and MICROSPORIDIA. Since the two subdivisions established by Schaudinn were strikingly different from each other, Hartmann (1907) separated CNIDOSPORIDIA entirely from SPOROZOA and gave the former a rank equal to the latter. Some modern protozoölogists, for example, Wenyon (1926), follow this scheme, while others (Calkins, 1926, etc.) the older system. Regardless of their taxonomic status, MYXOSPORIDIA, MICROSPORIDIA and ACTINOMYXIDIA are so closely related to one another that they all produce spores which are of unique structure, and Doflein's name CNIDOSPORIDIA is, therefore, indeed appropriate.

MYXOSPORIDIA have been known for nearly one hundred years and consequently a large number of papers were written about them by numerous observers and investigators. It is beyond the scope of the present chapter to consider the history of development of our knowledge about these organisms. The reader is referred to Gurley (1894), Thélohan (1895) or Auerbach (1910).

MYXOSPORIDIA seem primarily to be the parasites of lower vertebrates, especially of fishes. Nearly ninety-five per cent of known MYXOSPORIDIA are found in fishes, although they occur occasionally in reptiles and amphibians (Kudo, 1920). Every species of fish seems to harbor one species or another of this particular group of protozoa. As far as known, MYXOSPORIDIA were found everywhere in the world where microscopical examination of fishes has been carried. On several occasions, they were discovered by workers who were engaged in lines of research not connected with MYXOSPORIDIA, and this brought out numerous incomplete and confusing observations and descriptions concerning them. Fortunately, in the past twenty years there have been a number of zoölogists who have devoted themselves to the study of these organisms.

A great majority of known MYXOSPORIDIA have been observed in the fishes of France, Germany, Italy, Switzerland, England, the United States, Brazil, and Japan. It is hoped that re-examination of long known but little understood species occurring in Europe and America and further microscopical examination of fishes in other parts of the world would not only add some more taxonomic information, but also solve some of the problems set forth in the following pages.

CLASSIFICATION

Thélohan (1892) was the first to establish a classification of MYXOSPORIDIA. As numerous new species were discovered and as more information was added to the trophic phase of these PROTOZOA, a number of investigators attempted to improve the system. Auerbach (1910) gave an excellent summary of them. The writer (Kudo, 1920) also considered the systems then known and pointed out that "the suggestion as to the adoption of other characters than the spore for the divisions of MYXOSPORIDIA, proposed by Awerinzew (1907, 1908), Auerbach (1910) and Davis (1917), can only be applied in future. At the present time, the characters concerning the vegetative form do not appear to afford a better and more natural basis for the classification of MYXOSPORIDIA than those of the spore. Thus from the taxonomic point of view the present situation does not seem to be much improved as compared with that at the end of the last century."

Unfortunately this still holds true. In the last ten years, no one has undertaken to consider the system carefully, although Dunkerly (1925) made a brief remark with regard to the one the writer set down (1920). Both Calkins (1926) and Wenyon (1926) adopted the writer's scheme and included it in their treatises on protozoa. In the following, the system is revised in order to include three genera which were not included in it before:

Order MYXOSPORIDIA Bütschli, 1881[1]

Suborder EURYSPOREA Kudo, 1920
  Family CERATOMYXIDÆ Doflein, 1899
    Genus *Ceratomyxa* Thélohan, 1892
    Genus *Leptotheca* Thélohan, 1895
    Genus *Myxoproteus* Doflein, 1898 emend. Davis, 1917
    Genus *Wardia* Kudo, 1920
    Genus *Mitraspora* Fujita, 1912 emend. Kudo, 1920
Suborder SPHÆROSPOREA Kudo emend.–

Spores spherical or subspherical; one, two or four polar capsules. Sporoplasm without iodinophilous vacuole. Vegetative form found in organcavities and tissues. In marine and fresh-water fish and amphibians.

  Family CHLOROMYXIDÆ Thélohan, 1892
    Genus *Chloromyxum* Mingazzini, 1890
  Family SPHÆROSPORIDÆ Davis, 1917 emend.–

Spores with one or two polar capsules. Monosporous, disporous, polysporous.

    Genus *Sphærospora* Thélohan, 1892
    Genus *Sinuolinea* Davis, 1917
    Genus *Unicapsula* Davis, 1924.

Spores with one polar capsule. Shell-valves asymmetrical. In the muscle cell of marine fish. Polysporous. Type and only species: *Unicapsula muscularis* Davis, 1924.

Suborder PLATYSPOREA Kudo, 1920 emend.–

Sutural plane of the spore coincides with or at an acute angle to the longest diameter. One, two or four polar capsules. Sporoplasm with or without iodinophilous vacuole.

  Family MYXIDIIDÆ Thélohan, 1892
    Genus *Myxidium* Bütschli, 1882
    Genus *Sphæromyxa* Thélohan, 1892
    Genus *Zschokkella* Auerbach, 1910
  Family COCCOMYXIDÆ Léger et Hesse, 1907.–

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[1] For definitions of these groups not mentioned here, the reader is referred to Kudo (1920).

Spores ellipsoidal; circular in cross-section. With one polar capsule; without iodinophilous vacuole.

Genus *Coccomyxa* Léger et Hesse, 1907.–

Polar filament long. In organ-cavity of marine fish. Monosporous and polysporous. Type species: *Coccomyxa morovi* Léger et Hesse.[2]

Family MYXOSOMATIDÆ Poche, 1913 emend.–

Spores with two or four polar capsules at anterior end; with or without posterior prolongation of the shell-valve. Sporoplasm without iodinophilous vacuole.

Genus *Myxosoma* Thélohan, 1892
Genus *Lentospora* Plehn, 1905
Genus *Agarella* Dunkerly, 1915

Spores elongated ovoid; four polar capsules at anterior end. Posterior end of shell-valves prolonged into long processes. Polysporous. In testis of South American lung-fish. Type and only species: *Agarella gracilis* Dunkerly.[3]

Family MYXOBOLIDÆ Thélohan, 1892
Genus *Myxobolus* Bütschli, 1882
Genus *Henneguya* Thélohan, 1892
Genus *Hoferellus* Berg, 1898

[2] Recent studies by Dunkerly (1925) and Georgévitch (1926) indicate that this genus should be included in MYXOSPORIDIA.

[3] Dunkerly (1915) held the number of polar capsules as a more important character than the form of spore, and placed this genus in the family CHLOROMYXIDÆ. In a later paper (1925) in which he gave neither side view nor thickness of the spore, Dunkerly writes that "in Kudo's classification *Agarella* would come under the suborder SPHÆROSPOREA (an unfortunate name, since many of the included genera are anything but spherical in shape), and in the family CHLOROMYXIDÆ, the genus *Chloromyxum* being the only one known hitherto with four polar capsules." This is due to misunderstanding on the part of Dunkerly. Since Dunkerly states that "the form of the spore exactly resembles that of *Henneguya*" and that the spore was "slightly flattened in the sutural plane," *Agarella* is clearly to be considered a platysporid myxosporidian with four polar capsules, and not a spherosporid as Dunkerly designated. This is clearly to be seen from the definitions of these suborders (Kudo, 1920: 57). In MICROSPORIDIA (Kudo, 1924), the writer placed the number of polar capsules above the form of spore; in MYXOSPORIDIA, the form of the spore can be used with less confusion as the basis of establishing the major subdivisions of the group than the number of polar capsule. Since Dunkerly did not mention the presence of iodinophilous vacuole in the spore, it is supposed that this was absent. For these reasons the genus is placed in the family with a slight change in the definition of the family.

THE SPORE

As was pointed out briefly in the last section, the spore is still the most important stage of a myxosporidian from taxonomic point of view; in other words, in order to establish the myxosporidian nature and subsequent generic and specific identification, one must observe the spore. In this section, the structure of myxosporidian spores will be considered.

The spore of MYXOSPORIDIA is covered by a shell or spore membrane which is composed of two valves which come in contact in the sutural plane and which are usually symmetrical in form and similar in size. While in some species of *Chloromyxum,* the sutural line which marks the sutural plane, may be very indistinct, it is, as a rule, quite distinct and straight; in the genera *Sinuolinea* and *Unicapsula*, it may be sinuous. The margin of each valve is more or less thickened, which results in the formation of the sutural ridge. The shell-valve is usually uniform in thickness; in some species of the genus *Myxobolus*, however, it may differ considerably in different parts of the valve. The surface of the shell may be smooth or may exhibit various markings. More or less conspicuous ridges varying in number and form in different species, may run parallel to the sutural line, may show a network-like structure or may exhibit short tooth-like processes arising from the sutural ridge and radiating toward the center of each valve. When the ridges are fine, they form delicate striations, arranged usually parallel to the sutural line. Though these markings are ordinarily seen *in vivo,* they are very often readily studied in stained preparations.

The form of the spore varies greatly owing to the difference in the shell-valves together with variously developed appendages: (1) lateral appendages as in *Ceratomyxa;* (2) anterior processes as in *Myxoproteus;* (3) posterior processes as in *Wardia* (fringe-like), *Mitraspora* (filiform), *Hoferellus* (spinous), *Henneguya* and *Agarella* (tail-like), etc.

Inside of the spore membrane are present one, two or four polar capsules and usually one sporoplasm. Gurley (1894) and Davis (1917) used the term "capsules" instead of the polar capsules. It is, however, better to use the old term, in view of the fact that these polar capsules are situated at or near the more or less attenuated anterior end in the great majority of genera and species or at or near each extremity of the spore as in the family MYXIDIIDÆ. In a few species of some eurysporid genera such as *Wardia,* the polar capsules are not near any end, but even in such a case, the foramina of the polar filaments are located at the anterior end.

The polar capsules may be pyriform or spherical in form. They are situated at or near the anterior end of the spore. In MYXIDIIDÆ one polar capsule is situated at each end, in which case no distinction is to be made between the anterior and posterior ends. The number of the polar capsule in a spore, varies according to different genera. There is only a single polar capsule in the spore of unicapsular *Myxobolus, Coccomyxa* and *Unicapsula,* four in *Chloromyxum* and *Agarella* and two in all other genera. They may be equal or unequal in size and form within one and the same spore, although, as a rule, the former is the case. When two polar capsules are located at the anterior end, they may be convergent or divergent. Each has a foramen through the spore membrane in or near the sutural plane; occasionally the foramina may be so closely located that they form one foramen as in the case of a number of *Myxobolus.* Although the foramen is visible in fresh spores, it often becomes more distinct when the spores are stained. Each polar capsule contains a coiled polar filament which in most species can be recognized without difficulty in the fresh condition. The polar filament is, as a rule, a more or less long, most probably hollow thread connected with the polar capsule. The filament is extruded from the polar capsule through the foramen under the influence of the stimulants such as the digestive fluid of the host fish or certain chemicals (see p. 314). The filament is coiled along the inner surface of the polar capsule, except in *Sphæromyxa* in which the distal portion of the filament makes the central axis with the remaining part coiled around it.

Each spore possesses, when mature, a single sporoplasm, except in a few species in which there are two sporoplasms even in the mature spore (see p. 312). The sporoplasm occupies the extracapsular cavity of the spore and is of usually granular structure with two nuclei which are occasionally faintly visible in fresh spores. In the family MYXOBOLIDÆ, there is an iodinophilous vacuole mostly round or oval, which occurs in mature spores and which is one of the important characters of the family. Small refringent fat globules have also been seen in the spore.

Various axial nomenclatures which have been suggested and used by different authors, have led in many cases to confusion in descriptions. The writer has defined a group of terms necessary for accurate understanding of the spore (Kudo, 1920), which has been used also by Calkins (1926) and Wenyon (1926).

There are from one to four polar capsules at one end of the spore except in the family MYXIDIIDÆ and genus *Wardia*. This end is called the *anterior end*, and the opposite, the *posterior end*. The axis connecting these two ends is the *length* of the spore. In the case of *Leptotheca* or *Ceratomyxa*, *sutural diameter* is used in its place, since the breadth exceeds the length. The larger diameter of the spore measured at right angles to the length or sutural diameter is the *breadth*; and the smaller one the *thickness* of the spore. In the family MYXIDIIDÆ, there is a polar capsule at each pole; the axis connecting the poles is taken as the length; the larger diameter at right angles to it is the breadth, while the smaller one the thickness. In spores with posterior projections, the length of the spore is the distance between the anterior end and the inner margin of the posterior end of intrasporal cavity and the distance between the latter to the end of the tail or prolongation is the length of the tail. The sum of the two makes the *total length* of the spore.

The *front view* of a spore is the one in which its length and breadth are visible; the *side view* is the view in which the length and thickness lie horizontally; the *end view* is the one in which the breadth and thickness are observable.

MATERIAL

Although MYXOSPORIDIA inhabit various parts of the host body, they could conveniently be divided into those which inhabit the organ-cavities of the host and those which attack the host's tissues or cells. The latter forms (histozoic or cytozoic MYXOSPORIDIA) frequently produce conspicuous pathological changes in the host and have been discovered on numerous occasions by persons engaged in other studies of the fish.

When tissues near the body surface or of the fin, are invaded by MYXOSPORIDIA, the infected lesions may show striking growths of tumors. An examination of a very small portion of their contents would usually reveal an enormous number of spores. The gills are very frequently infected and, aside from a few cases of general infection, the myxosporidian invasion is manifested by the presence of tumors, cysts or growths on them. The latter are usually milky white and vary in size and form. A number of species have been found to inhabit superficial parts of the integument, namely, on or under the scales. In such cases the size of the myxosporidian body seems to be limited by that of the scale. This is the case with *Myxobolus squamæ* which Keysselitz (1908) found in the barbel. The writer has in his possession two specimens of river-chub, *Hybopsis kentuckiensis,* of Illinois, which exhibit a similar condition.

MYXOSPORIDIA are also frequent parasites in the tissues of various internal organs of the host fish. They have been found in the heart, swim-bladder, nervous system, connective and supporting tissues, muscles, various parts and glands of the digestive system, spleen, gonads, excretory system and mesentery. Here also, if the myxosporidian infection is at an advanced stage, one will find abnormal local enlargements or growths which are usually of milky white coloration.

The organ-cavity inhabiting forms (cœlozoic MYXOSPORIDIA) are either freely suspended in the fluid or attached to the epithelium of the organ. The gall-bladder is most frequently inhabited by the species of genera *Leptotheca, Ceratomyxa, Chloromyxum, Myxidium, Sphæromyxa* and *Zschokkella.* The uriniferous tubules of the kidney and the urinary bladder are also frequently the site of infection by cœlozoic MYXOSPORIDIA. Ordinarily no external changes accompany the presence of MYXOSPORIDIA living in the host's organ-cavity. There is no symptom by which one can suspect a myxosporidian infection. But the gall bladder of *Trutta fario* heavily infected by *Chloromyxum truttæ* was noted by Léger (1906) to be highly enlarged and appeared reddish yellow in color. The adjacent organs were deeply stained by the bile. As a rule, however, one has to subject the bile or the contents of the uriniferous tubules of the kidney or of the urinary bladder to microscopical examination in order to find the organisms.

As to the sources of material, the common fresh-water or marine fishes should be examined. For cœlozoic forms, their gall bladder, kidney and urinary bladder must be examined. The kidneys of *Rana clamitans* and *R. pipiens* harbor frequently *Leptotheca ohlmacheri* (Kudo, 1922). For histozoic forms, various tissues of the common fresh-water fishes should be examined. For further specific host-parasite relations, the reader is referred to Kudo (1920).

METHODS

Fresh Material. As far as is possible, careful examination of fresh material should be undertaken. In the case of a histozoic myxosporidian which forms tumors, cysts or growths on the body surface or in the internal organs of the host, note carefully their location, form, size and color. By cutting or pricking the surface of the lesion slightly, obtain a small amount of material which is ordinarily composed of soft, homogeneous and milky white amorphous substance (the spores), and make an ordinary fresh preparation, by adding, if necessary, a drop or two of the physiological salt solution. If the pathological changes observed on the host's body are due to a myxosporidian infection, one would most likely find a more or less large number of small refractile bodies, the spores.

In order to make an accurate observation of fresh spores, it is, however, better to make hanging drop preparations which were improved upon recently by Nemeczek (*1926*). In this, instead of one coverglass, two are used; the upper square one should be big enough to cover the entire hollow of the slide and the lower circular one small enough not to touch the margin of the hollow. A small amount of the material is put on the center of a large coverglass on the margin of which a line of vaseline is smeared, cover the drop with a small circular coverglass and over the whole place a depression slide so as not to allow the smaller coverglass to come in contact with the margin of the hollow of the slide. Press down slightly and make the larger coverglass adhere to the slide. By carefully and quickly turning the preparation over, examine it coverglass up. This preparation is much better than either an ordinary fresh preparation or hanging drop preparation, since (*1*) the spores are free from mechanical pressure and stationary, and (*2*) the material is in a uniformly thin film instead of a droplet as is always the case in a hanging drop preparation. Hence, an oil immersion objective is easily applied for observation as well as for drawing with camera lucida.

Make observation first with an objective with medium magnifying power, and then with an oil immersion objective and a fairly high-power ocular which combination would give a magnification of at least 1000 times the natural size. Note the general appearance of the spore. Measure the length, breadth and thickness of the mature spores which are distinctly outlined and which are the representatives of the group. Note the number and measure the length and largest diameter of the polar capsules. These measurements should be made on as many spores as possible, taken at random from numerous fields; one hundred spores would give fairly representative dimensions. In each of the polar capsules, one should notice a beautifully coiled polar filament which is almost always visible without the aid of any reagent. By counting the number, and measuring the diameter of coils of the polar filament, one can calculate the length of the polar filament without causing its extrusion.

An ordinary preparation may contain some spores with extruded polar filaments, which is probably due either to mechanical pressure during the preparation or to a change in the medium. The polar filaments of fresh spores are easily made to protrude from the latter by addition to the preparation of a dilute solution of mineral acids, potassium or sodium hydrate, ammoniacal water, glycerin, alcohol or ether, according to various workers. Potassium hydrate in ten to thirty-five per cent solution has been satisfactorily used by the writer in various genera and species. Hydrogen peroxide, the gastric and intestinal fluid of the host animal and mechanical pressure as applied on the coverglass also cause the filament extrusion. Spores which were smeared and dried on a coverglass or slide extrude their polar filaments upon addition of tap or distilled water according to Auerbach (1910) and others.

Observe the striations, ridges or other markings on the spore membrane. The sutural line or ridge in relation to the polar capsules should be made out carefully.

In the posterior half of the spore, one will find a sporoplasm which is more or less well defined. In a few species, for example *Leptotheca ohlmacheri,* two sporoplasms are regularly present in a young as well as a mature spore. In the sporoplasm, notice several granules, In some cases, the nuclei, ordinarily two in number, will be found; the endosome of each of the nuclei is conspicuous and surrounded by a ring-like nuclear membrane. In the spores of the family MYXOBOLIDÆ, there is a comparatively large vacuole which, upon addition of Lugol's solution, stains reddish orange in color. This is the iodinophilous vacuole.

If a part of an organ such as liver, kidney, spleen, etc., is to be examined, cut a small piece; by adding a drop of physiological salt solution, tease it out by means of dissecting needles and make a preparation as stated above.

If one looks for cœlozoic forms inhabiting the gall bladder or urinary bladder, examine the contents to find the floating forms and the scrapings from the epithelium of the organ to find the attached forms.

Besides the spore, one should notice developmental stages or trophozoites. In a histozoic form, there are usually present in the preparation numbers of developmental stages of sporoblasts or pansporoblasts with varying number of nuclei; in cœlozoic forms, small or large trophozoites, some of the latter contain developing or mature spores, occur ordinarily in the fluid of the organ under examination.

For taxonomic purposes, the data thus obtained will, together with the specific name of the host fish, enable one to determine the genus to which the myxosporidian belongs and in many cases the specific name by comparing with the previously known species. All the genera and species which had been known up to 1919, with the exception of *Coccomyxa* and *Agarella*, will be found in Kudo (1920).

Preserved Material. If through the examination of fresh material as outlined above, the myxosporidian nature of the parasite is established, make several smears on coverglasses of the material taken from the lesion, and fix them while wet with good fixatives. Ordinarily Schaudinn's, Bouin's, Carnoy's or Flemming's is used with satisfaction. It is desirable to use a number of different fixatives as well as different staining methods.

For staining, Heidenhain's iron hematoxylin, Delafield's hematoxylin, Dobell's alcoholic iron hematein, Giemsa's stain, etc., are used. When stained with Giemsa, stain with a dilute solution (one drop to one cubic centimeter of neutral distilled water) overnight with the smeared surface down, wash with distilled water and pass through pure acetone, mixtures of acetone and xylol of various proportions and finally xylol. Mount the smears in cedar oil.

The remaining part of the material should be fixed *in toto* and sectioned, some slides with uniformly thin (three microns) and others with uniformly thick (six to ten microns) sections, in order to study the relation between the parasite and the host body and also the structure of the parasite itself.

The stained smears should next be studied. Make a large number of drawings, showing the front, side and end views of mature spores, and also developmental stages which will be more clearly noticed in this condition than in the fresh state. Measure the spores. Cépède (1906) first called attention to the fact that fresh spores and fixed, stained and mounted spores differ in size and justly insisted that for taxonomic purposes measurements of the spore should be accompanied by a statement of the condition—whether fresh or fixed—in which the spores were measured. For *Myxobolus cycloides,* Cépède gave the following dimensions: fresh spores: length 13.5-16μ; breadth 11-13μ; mounted spores (Schaudinn, Heidenhain, balsam): length 10.5-12μ; breadth 7.5-8μ. Thus the length and breadth of the spore decreased by twenty-five and thirty-five per cent, respectively, by fixation and mounting. The writer also pointed out the importance of the matter (Kudo, 1921*a*) and showed that in the case of *Leptotheca ohlmacheri* the decrease in dimensions of fixed spores was due to shrinking of the entire spore. Various fixatives and staining methods were used for both smears and sections. The sutural diameter, breadth and thickness of fixed spores were approximately 16, 28 to 29 and 17 to 21%, respectively, smaller than those of fresh spores. For this reason, one must use care in comparing the dimensions of spores under observation with those of previously known species which have been observed under various circumstances.

Frequently one has to study a specimen which has already been preserved in alcohol or formol, the most commonly employed preservatives. A part of the cyst or the organ of the host fish, is removed on a slide and washed in distilled water. A number of improved hanging drop preparations should be made from it and studied in a way similar to that which was described for fresh material.

Extrusion of the polar filaments from preserved spores does not ordinarily take place with the methods effective for fresh spores. Gurley (1894), however, stated that in alcoholic specimens, spores occasionally extruded their polar filaments under the action of sulphuric acid or iodine. Almost all of the freshly preserved spores may extrude their polar filaments. Kudo (1920) fixed the gills of *Carpiodes difformis* which carried a number of cysts of *Myxobolus discrepans* in Carnoy's alcohol-acetic for twenty-four hours and preserved them in ninety-five per cent alcohol.

For thirty days, almost all the spores thus preserved were capable of filament extrusion upon addition of a drop of potassium hydrate solution (thirty-five per cent). At the end of two months, complete filament extrusion was noted in numerous spores when treated in a similar manner. From that time on, the number of spores which were able to protrude their polar filaments decreased, and at the end of nearly five months, about fifty per cent of the spores examined, extruded "short and not fully extended" filaments under the influence of the potassium hydrate solution. In preserved spores, the length of the polar filament should, therefore, be better calculated by counting the number and measuring the diameters of the coils of the filament within the polar capsule.

The remaining part of the material should be made into smears and sections and studied as was stated above for the fresh material.

PROBLEMS

1. *Improved classification.* The present system of classification of the MYXOSPORIDIA, as outlined elsewhere, appears to be better fitted for our present state of knowledge of this group than any other (Kudo, 1920; Calkins, 1926; Wenyon, 1926). Increase of our knowledge of these organisms would without doubt result in bringing out a better and more natural classification than the present one.

2. *Phylogeny of the Myxosporidia.* Some attempts were made to consider the phylogenetic relation of MYXOSPORIDIA to other groups of animal kingdom. Emery (1909), Ikeda (1912) and others, pointed out that the spore of MYXOSPORIDIA and especially of ACTINOMYXIDIA, an allied group, resembles the ciliated stage of *Dicyema*, a mesozoan, in that each possesses protective cells within which are present reproductive cells.

3. *Iodinophilous vacuole.* The writer treated the spores of three species of *Myxobolus* and of two species of *Henneguya* with various glycogen-testing methods, and came to the conclusion that the vacuole contained a substance whose chemical reactions were similar to those of glycogen (Kudo, 1921). It is generally understood without any experimental evidence that the glycogen is probably used in the future development of the sporoplasm.

4. *Status of various genera.* The existing genera are not well differentiated. The genera *Myxosoma* and *Lentospora* should possibly be united under the former, since the difference noted be-

tween them seems to be too small for generic distinction. On the other hand, the unicapsulated and bicapsulated *Myxobolus* should better be separated into two different genera.

5. *Artificial cultivation.* Although a number of investigators have observed certain changes which myxosporidian spores undergo when subjected to the digestive fluid of the host fish, no one has succeeded in cultivating a myxosporidian *in vitro*.

6. *Problems concerning infection through the digestive tract.* Thélohan (1895) established the fact that the spores of *Henneguya psorospermica* underwent certain changes when the gill-filaments of infected perch containing the myxosporidian cysts were introduced into the alimentary canal of another host fish, and considered that the infection takes place through this system of the fish. Subsequent observers confirmed this in various species of MYXOSPORIDIA.

Auerbach (1909) undertook careful infection experiments with *Myxidium bergense* on young *Gadus virens*. He noted that young amœbulæ which emerged from the spores in the duodenum of the host proceeded immediately up the bile duct, and after leading intracellular life in the epithelium of the bladder or of the proximal part of the duct, become extracellular stages which inhabited the gall bladder. Erdmann (1912, 1917) fed the host fish the spores of *Chloromyxum leydigi* in gelatine capsules and found that two-thirds of thirty-three fish thus fed became infected. In from three to five days after the feeding, numerous trophozoites are said to have appeared in the host's gall bladders. Davis (1916) observed the emergence of the sporoplasm as an amœbula in five to fifteen minutes from some of the spores of *Sinuolinea dimorpha* which were subjected to the fluid taken from the pyloric ceca of the host fish. Similar observation was recorded by Georgévitch (1917). Kudo (1922) treated the spores of *Leptotheca ohlmacheri* with the digestive fluid of the frog and observed the emergence of the sporoplasms as amœbulæ. Similar changes were noticed in the spores which were introduced into the digestive tract of the host.

The fact that prior to the emergence of the sporoplasm from the spore the polar filaments are extruded from the capsules and the spore itself, and shell-valves become opened along the sutural plane, has been established in numerous species. As to the function of the filament, very little is known at the present time. Leuckart (1879) suggested that the polar filament is an attachment apparatus. Bütschli (1882), Thélohan (1895), Doflein (1899) and others have expressed similar views. A thorough comprehension of the mechanism of extrusion and of significance awaits future investigations.

As to the further changes of amœbulæ within the host body, our knowledge is fragmentary, since there seems to be little convincing experimental evidence. All authors seem to agree in maintaining that the liberated amœbulæ in some way penetrate the gut-epithelium and enter blood or lymph vessels. With the circulation of the blood or lymph, they are carried into specific sites of infection within the host body. In the case of MYXOSPORIDIA inhabiting the urinary bladder of the host, some investigators hold the view that the amœbulæ make their way to the bladder directly through the ureter (Davis, 1916). No one has succeeded yet in tracing by experimental methods the entire change—from the emergence of the amœbulæ up to their establishment in specific sites within the host body. The factors involved in the germination of the sporoplasm and, further, the factors which delimit numerous species of MYXOSPORIDIA to a limited specific site of infection in the host body, if the amœbulæ make their way passively with the blood or lymph stream of the host, await future researches.

7. *Problems concerning other modes of infection.* Although there is at least one case of well-established "hereditary infection" in MICROSPORIDIA (see p. 340), no myxosporidian has been seen to infect the next generation of the host through the germ cells. That this mode of infection may occur in MYXOSPORIDIA is highly probable in view of the fact that several species were found infecting ovarian eggs of certain fish (Kudo, 1920).

8. *Viability of the myxosporidian spore.* As to how long the myxosporidian spore would live under various circumstances, we know very little. In order to determine whether the spores are alive or not, one has to depend upon feeding experiments with the host fish, since even the emergence of the sporoplasm in the digestive fluid does not necessarily indicate the capability of the amœbula to invade the host's tissue or organs and establish an infection.

9. *Behavior of the two nuclei of the sporoplasm prior to, at the time of or shortly after the latter's emergence from the spore.* In the great majority of MYXOSPORIDIA there is a single binucleated sporoplasm in each spore.

Concerning the behavior of these two nuclei of the sporoplasm there have been a number of observations which may be put under the following groups:

a) *The two nuclei fuse into one.* Several investigators noticed one or two nuclei in the sporoplasm of the spores found in a single cyst of a myxosporidian (Schröder, 1907, Keysselitz, 1908, Mercier, 1909, Auerbach, 1910, Erdmann, 1917, Georgévitch, 1915, Kudo, 1922, 1926, etc.). The spore containing the sporoplasm with a large nucleus is considered by these investigators as more mature than that with binucleated sporoplasm, that is to say, the two nuclei of the sporoplasm are considered to have fused with each other.

b) *The binucleate sporoplasm divides into two uninucleate amœbulæ and there is no fusion of the nuclei.* Schuurmans-Stekhoven (1919) who studied *Myxobolus swellengrebeli* proposed this view, which if it really occurs in this species appears to be a unique process, since no other observer has agreed with the view. The same investigator (1920) does not seem to uphold this view in his study of *Myxobolus destruens.*

10. *Problems concerning the nuclei of the trophozoite.* All investigators agree that the youngest trophozoite is a uninucleate form. The nucleus undergoes multiplication. In the well-studied forms, there are two types of nucleus, vegetative and generative. Mavor (1916), Davis (1916) and Kudo (1922), all working on cœlozoic forms, noted that the first division of the trophozoite nucleus results in production of a generative and a vegetative nucleus. In the polysporous histozoic MYXOSPORIDIA, the two types of the nucleus have also been noted, but their early history starting with the nucleus in the uninucleate trophozoite awaits future investigations.

As to the transformation of the vegetative nucleus to the generative, there are two views. Davis (1916, 1923) and Bremer (1922) hold that the vegetative nucleus does not give rise to the generative nucleus, after it has differentiated as such, while Schröder (1907), Lo Giudice (1912), Georgévitch (1917), Kudo (1922, 1926) and others maintain that the vegetative nucleus gives rise to the generative nucleus by further divisions. According to Debaisieux (1924, 1925), two vegetative nuclei fuse to form a generative nucleus. All agree, however, that the vegetative nucleus divides. The division appears to be amitotic according to Kudo (1922, 1926), Davis (1923) and Debaisieux (1924, 1925).

The fact that the generative nucleus divides by mitosis, has been known since Thélohan's work. The process has been observed by subsequent investigators in both cœlozoic and histozoic species.

The trophozoites multiply in number. In cœlozoic forms, plasmotomy (Doflein, 1898) has been noted to occur in several species (Laveran and Mesnil, 1902, Georgévitch, 1917, 1919, etc.). Multiplication by budding or gemmation has also been noted by Cohn (1895), Davis (1916), Georgévitch (1917), Kudo (1922), Bremer (1922), and others. In the histozoic form, the trophozoite is considered as capable of multiplication, but exact information as to how and when this occurs is still lacking.

11. *Problems concerning the origin of the sporoblast or pansporoblast.* All investigators are agreed that the earliest developmental stage is a uninucleate amœbula found in the intestine or blood stream of the host body. As to the origin of the pansporoblast formation, there are many and diverse views, which may be put under the following general groups:

a) Each generative nucleus becomes a sporoblast or a pansporoblast by being surrounded by an island of cytoplasm and the nucleus gives rise by repeated divisions to several nuclei to form one or two spores.

b) A pansporoblast is formed by union of two cells. In this, three groups of opinion are to be distinguished:

(1) By union of two uninucleate gametes.

(2) By union of two binucleate cells, the nuclei of each of which were formed by a division of a single nucleus.

(3) By union of two binucleate cells, each of which was formed by cytoplasmic fusion of two uninucleate bodies.

Up to the present time, no one has succeeded in observing the changes which take place in the trophozoite and which result in the formation of the sporoblast or pansporoblast in the living state and the views summarized above of various investigators were based exclusively upon careful studies of various phases as seen in permanent preparations.

12. *Problems concerning the developing sporoblast and pansporoblast.* In some forms in which a single generative nucleus gives rise to a single sporoblast, the nucleus presumably divides into two; one small nucleus remains as the vegetative nucleus of the sporoblast, while the large nucleus divides repeatedly to produce from six (in bicapsulated spore) to eight (in tetracapsu-

lated spore) (see, for example, Kudo, 1922). Georgévitch (1919) notes that the vegetative nucleus may divide once to form two nuclei. In a developing sporoblast of all forms there were noted two nuclei for the spore membrane, two for the sporoplasm or sporoplasms, and as many nuclei as there are developing polar capsules. In the case of a disporoblastic pansporoblast, the smaller nucleus divides once according to the investigators who hold the view 11-a and forms two vegetative nuclei of the pansporoblast. The large nucleus divides repeatedly, giving rise to from ten or twelve to sixteen nuclei. Two spores are thus formed; in the meantime the two vegetative nuclei degenerate with the development of the spores.

According to the investigators who hold the view 11-b-1, the zygote nucleus gives rise to two "reduction nuclei" (vegetative nuclei) and sixteen nuclei which develop into two bicapsular spores. On the other hand, workers who hold the views 11-b-2 or 11-b-3, assume that the two small nuclei remain as vegetative nuclei of the pansporoblast and each of the other two large nuclei gives rise by division to a definite number of nuclei to form a spore.

Besides these two pansporoblast nuclei, several authors note the appearance of compact bodies which take nuclear stains deeply, in the developing sporoblast or pansporoblast. Erdmann (1917) was inclined to think them glycogen bodies which are used for the formation of the spore membrane and polar filaments. Kudo (1923, 1926) observed that these bodies appear as the amount of the endosome (plasmosome) of the dividing nuclei decreases and that on a number of occasions the endosome appeared to be just extruded from the nucleus through the nuclear membrane. He considered it probable that the endosomes of the nuclei of the developing sporoblasts are extruded to the periphery of the cytoplasmic body and used for the formation of the spore membrane.

13. *Pathological.* There is lack of precise information concerning the pathological effect of MYXOSPORIDIA upon the host body. Various investigators report in numerous instances remarkable changes which were supposed to be due primarily to infections of certain MYXOSPORIDIA, but in most cases they could not show conclusively that the changes were due exclusively to MYXOSPORIDIA. Observations up to 1893 are excellently described by Gurley (1894), and Thélohan's monograph (1895) contains useful ob-

servations on this point. Here a few of the more recent findings will be considered.

1) *Effect of the cœlozoic Myxosporidia upon the host body.* Auerbach (1910) considered through infection experiments that there was an intracellular stage in *Myxidium bergense* during its early trophic life. Exact information in regard to this observation is lacking. The majority of investigators have not noted any pathological effects on the parasites upon the host body. In the case of a heavy infection, remarkable changes have been reported to occur. Léger (1906) noted that a heavy infection by *Chloromyxum truttæ* of the gall bladder of *Trutta fario* resulted in a chronic disease among the host fish in France. The infected fish lose appetite, the fecal matter becomes diarrheic, and the entire body anemic. In many cases the fish succumbed to the disease. Upon autopsy it was found that the liver was decolorized, the gall bladder was enormously distended, and the bile was yellowish red in color. The muscles and abdominal wall were deeply colored by the bile. Léger attributed these changes to the presence of enormous numbers of trophozoites of the myxosporidian in the gall bladder and bile duct of the infected fish.

Auerbach (1909) noticed a highly thickened epithelium of the gall bladder of *Gadus virens* which was infected by *Myxidium bergense*, which he compared with a cystitis. According to Doflein (1898), when each of the large intracellular stages of *Hoferellus cyprini* which occur in the uriniferous tubules of the kidney of the carp, leaves the host cell, the latter secrets certain substances and transforms itself into a "yellow body" and when a large number of the latter bodies are present in the host fish death results.

Ohlmacher (1893) and Whinery (1893) noted that the kidneys of *Bufo lentiginosus* infected by *Leptotheca ohlmacheri* were enlarged and in a congested state. The same myxosporidian was found in *Rana clamitans* and *R. pipiens* by Kudo (1922) who noted that the infected kidneys were slightly larger than the normal ones and were usually greatly congested.

2) *Effect of the histozoic Myxosporidia upon the host body.* MYXOSPORIDIA have been reported from every organ or tissue of the fish, although as a rule a species confines itself to a specific tissue of a specific fish. Numerous instances of distinctly recognizable ill effects of histozoic MYXOSPORIDIA upon the host body have been reported. A heavy infection of the greater part of the

gills by *Myxobolus* or *Henneguya* apparently disturbs the respiratory function of this organ in such a way that the host fish succumbs to the infection. The muscles are the seat of infection of several species of MYXOSPORIDIA. Perhaps the most thoroughly studied form is *Myxobolus pfeifferi* which attacks the muscles and connective tissue of various organs of the barbel and other freshwater fishes in European waters. Conspicuous boils appear in heavily infected fish, hence this myxosporidiosis is known as "boil disease" (Pfeiffer, Thélohan, Keysselitz, etc.). The boils are principally located in the body muscle which condition hinders the movement of the infected fish. When the boils become large, the color and luster of the body surface are lost, the scales fall off, and the host finally dies. At the beginning of the infection, the muscles appear yellowish in color and are soft and gelatinous. Such a fish has a bitter taste when cooked, and hence has no commercial value. According to Thélohan and Keysselitz, the direct change which the infected muscle fiber manifests, is a hyaline degeneration of the fibrils. Davis (1924) recently finds that the so-called "wormy" halibut along the Pacific coast of North America is due to an infection of the muscle fibers by an intracellular myxosporidian, *Unicapsula muscularis*. Elongated vegetative forms of this myxosporidian are found to be present in a single row within the entire length of the fiber. They, however, do not extend to the sarcolemma, "there being always a thin layer of sarcoplasm and muscle fibrils between the two." Davis found that the muscle fibers showed little sign of injury in spite of the abundance of the parasites. But the infected fibrils become swollen and undergo a hyaline degeneration; no fibers have, however, been observed in which fibrils were entirely destroyed.

The circular muscle fibers of the small intestine of a black crappie which were in direct contact with the cysts of *Myxobolus intestinalis* were seen by the writer (Kudo, 1929) to become separated from one another and to turn about ninety degrees from their original axial direction. These fibers become undulating fibrillar structures which later degenerate completely.

Lentospora cerebralis is now generally recognized as the causative organism of "Drehkrankheit" of SALMONIDÆ and GADIDÆ through the studies of Hofer and Plehn (1904, 1924). According to Plehn, young fish are particularly susceptible to infection. The posterior region of an infected fish loses its normal arrangement of chromatophores and becomes darkly colored. The movement of such a fish is greatly affected. Since the myxosporidian attacks the cartilage of the auditory organ, the infected fish loses its sense of equilibrium and this together with the twisted hind part of the body results in a very peculiar turning movement of the host fish.

Pimephales notatus of North America is strangely susceptible to an infection of *Myxobolus notatus* (Mavor, 1916, Kudo, 1929). Debaisieux (1925) found an apparently similar species in *Leuciscus rutilus* of Belgium. In all cases the myxosporidian attacks the subdermal connective tissue of the host fish only. The infected tissue shows an abundance of blood capillaries. The tissue which is in direct contact with the parasite appears to be an epithelial tissue which was noted by all three workers. As to the origin of this apparent "cell-layer," the writer showed that it is modified connective tissue cells of the host, which change took place as the result of myxosporidian stimulation (Kudo, 1929).

14. *Problems of "diffuse infiltration."* In numerous histozoic MYXOSPORIDIA, there have been noted besides the cysts a number of spores scattered in the adjacent connective tissue; *i.e.,* the host cells and myxosporidian spores intermingle with each other. Thélohan (1895) noted this state and called it "parasitic" or "diffuse infiltration." With regard to the modes of formation and significance of such diffused state of infection in histozoic MYXOSPORIDIA, the main foci of infection of which are manifest in cystic form, a number of opinions were advanced by Thélohan, Keysselitz (1908), Auerbach (1912), Schuurmans-Stekhoven (1920), Kudo (1926), etc. A better comprehension of this condition must depend on future investigations.

15. *Problems of autoinfection.* As to whether or not the spores would germinate in the host body in which they developed or, in other words, whether autoinfection of MYXOSPORIDIA occurs or not, there are two views: Pfeiffer (1888), Thélohan (1895), Georgévitch (1914), Kudo (1920, 1926), Debaisieux (1922) and others, considered that the spores would under certain circumstances germinate in the body of the host in which they developed and give rise to new infection. On the other hand, one may suppose that this is improbable since the germination of the myxosporidian spore in the host organ other than the alimentary canal has yet to be determined.

16. *Problems concerning seasonal occurrence.* The great majority of MYXOSPORIDIA were discovered during the warmer months of the year. This is easily understood as the material is more abundantly obtained and studied during this period. Do MYXOSPORIDIA occur in equal abundance in winter months as they do in summer months? Gurley (1894) wrote that *Myxobolus transovalis* which was first noted in June, could not be found in August in host fish which were collected from the locality where the previous collection had been made. Davis (1917) stated similarly that *Ceratomyxa monospora* was abundantly found in June, much less in July and disappeared completely from the host fish at the end of the month. Contrary to the above observations, Keysselitz (1908) observed that the infection by *Myxobolus pfeifferi* as manifested by the appearance of the boils on the barbel is not noticeable during winter and spring months. The boils appear in April and continue to be present up to the end of October, during which months the majority of the heavily infected fish die. The highest mortality is reached in the hottest months, *i.e.,* July and August. That the temperature affects the growth of the parasite was indicated by a rapid daily growth in size of the boils on fish which were kept in the aquarium at a temperature of 25° C. or higher.

Wegener (1910) observed *Henneguya psorospermica* throughout the year, but rarely during winter. According to Nemeczek (1911), the cysts of *Henneguya gigantea* do not, after October, contain the spore, but vegetative forms only. This observer holds as does Keysselitz that the development of the spore depends apparently upon the temperature of the water in which the host fish live. Zandt (1924) studied *Perca fluviatilis* of Lake Constance, Switzerland, for two years with regard to the infections by *Henneguya similis.* He found an annual periodic appearance of the myxosporidian. The infection was first noted in December and January, when mature spores are not found. The spores begin to appear from February to March and infected fish were observed up to about the end of May. From the latter time to December, no host fish carrying cysts were recognized.

These data are too meager to allow any definite conclusion. There seem to be numerous factors affecting the host-parasite relation in MYXOSPORIDIA as revealed by these apparently contradictory observations. In the bibliography, references which are listed in Kudo (1920) are omitted.

CHAPTER XXXIII

MICROSPORIDIA

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INTRODUCTION

The MICROSPORIDIA (Balbiani, 1882), together with MYXOSPORIDIA and ACTINOMYXIDIA, make up the group of PROTOZOA to which Doflein (1901) gave the name CNIDOSPORIDIA. Consequently the taxonomic status of this group is the same as that of the MYXOSPORIDIA (p. 304).MICROSPORIDIA have been known to science since the middle of the last century when the pébrine disease of the silkworm broke out in several European countries in a severe epidemic form. Because of the economic importance of this disease, numerous investigators directed their attention to the causative organism of the disease, a microsporidian, now known as *Nosema bombycis*, and this resulted in the appearance of a large number of papers dealing with this and allied forms discovered in various groups of animal belong to phylum ARTHROPODA. For the history of development of our knowledge on MICROSPORIDIA, the reader is referred to Auerbach (1910).

As is the case with the members of SPOROZOA, MICROSPORIDIA are all parasites. Unlike the MYXOSPORIDIA which are primarily fish-parasites, the MICROSPORIDIA have been found in invertebrates such as PROTOZOA, PLATYHELMINTHES, BRYOZOA, NEMATHELMINTHES, ANNELIDA and ARTHROPODA, and also in vertebrates such as PISCES, AMPHIBIA and REPTILIA. The great majority of host animals known up to date are arthropods and especially insects (Kudo, 1924). The MICROSPORIDIA have been reported from various regions of the world, particularly from France, Germany, the United States and Brazil. Up to 1924, 178 species of MICROSPORIDIA and at least 222 host species had been known, and these numbers have somewhat increased in the last five years.

CLASSIFICATION

After making a comparative study of several systems of classification of MICROSPORIDIA, the writer came to the conclusion that that proposed by Léger and Hesse (1922) fitted best to the state of our knowledge and adapted it (Kudo, 1924). Although numerous papers dealing with MICROSPORIDIA have appeared since that time, the system still remains the most satisfactory one. Unfortunately three generic names proposed by Léger and Hesse (1921, 1922) were found to be preoccupied. Their replacement by new names has not been well known and this has resulted in slight confusion. For this reason the entire system is here given:

Order MICROSPORIDIA Balbiani, 1882

- Suborder MONOCNIDEA Léger et Hesse, 1922
  - Family NOSEMATIDÆ Labbé, 1899
    - Genus *Nosema* Nägeli, 1857 emend. Pérez, 1905
    - Genus *Glugea* Thélohan, 1891 emend. Weissenberg, 1913
    - Genus *Perezia* Léger et Duboscq, 1909
    - Genus *Gurleya* Doflein, 1898
    - Genus *Thelohania* Henneguy, 1892
    - Genus *Stempellia* Léger et Hesse, 1910
    - Genus *Doboscqia* Pérez, 1908 emend. Kudo, 1924.
    - Genus *Plistophora* Gurley, 1893
  - Family COCCOSPORIDÆ[1] Kudo, 1925 (for family COCCONEMIDÆ Léger et Hesse, 1922)
    - Genus *Coccospora*[1] Kudo, 1925 (for genus *Cocconema* Léger et Hesse, 1921)
  - Family MRAZEKIDÆ Léger et Hesse, 1922
    - Genus *Mrazekia* Léger et Hesse, 1916
    - Genus *Octosporea* Flu, 1911 emend. Chatton et Krempf, 1911

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[1] Since Genus *Cocconema* Léger et Hesse was found by Dr. Cockerell to be preoccupied by *Cocconema* Ehrenberg, 1829, genus *Coccospora* and family COCCOSPORIDÆ were suggested in their place (Kudo, 1925).

Genus *Spiroglugea* Léger et Hesse, 1924 (for *Spironema* Léger et Hesse, 1922; syn. *Spirospora*[2] Kudo, 1925)
Genus *Toxoglugea* Léger et Hesse, 1924 (for *Toxonema* Léger et Hesse, 1922; syn. *Toxospora*[2] Kudo, 1925)
Suborder DICNIDEA Léger et Hesse, 1922
Family TELOMYXIDÆ Léger et Hesse, 1910
Genus *Telomyxa* Léger et Hesse, 1910

THE SPORE

As in the case of MYXOSPORIDIA, the spore is perhaps the most important stage of a microsporidian from the taxonomic standpoint, which fact is widely recognized. The vegetative stage of a microsporidian possesses without doubt its own characteristic appearance and structure, yet the present optical apparatus and technique do not allow one to determine the microsporidian nature of an organism unless one sees its spore stage. Aside from its taxonomic importance, the microsporidian spore possesses interest to the protozoölogist in that it is probably one of the smallest protozoan cells and it contains a unique structure, known as the polar filament, which is ordinarily long and fine. As in other spore-producing forms of microörganisms, the spore becomes the source of infection to new hosts which are often animals useful to man. It is, therefore, the most important phase in a microsporidian from the economic standpoint also. For this reason, one section for the spore will not be out of place.

The form of microsporidian spores varies somewhat among different genera and is used as the basis for the family differentiation. The great majority of the spores are oval, ovocylindrical or pyriform with various intermediate forms; in some cases, they may be spherical, reniform, tubular, bacilliform, crescentic, commaform or even spiral. Although there are certain species such as *Mrazekia mrazeki,* in which a diversity in form of spore was noted and also a number of species in which abnormal spores were noted to occur, the great majority of the spore of one and the same species are somewhat uniform in form with little variation.

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[2] Without knowledge of Léger and Hesse's paper (1924) in which these two authors proposed *Spiroglugea* and *Toxoglugea* respectively for *Spironema* and *Toxonema* which names had been preoccupied, the writer proposed to substitute the latter two by *Spirospora* and *Toxospora* (Kudo, 1925). These two names therefore become synonymous with *Spiroglugea* and *Toxoglugea* respectively. The writer wishes to thank Dr. C. W. Stiles for kind advice in this connection.

The size of the microsporidian spores also varies a great deal between wide extremes (1.25 by 1μ in *Nosema pulvis* and 17-23 by 3.5μ in *Mrazekia argoisi*), but the majority are less than ten microns in length.

In almost all cases, the mature spores of one and the same species occurring in a single host cell or tissue appear to vary in size to some extent. One, however, finds intermediate forms between the two extremes. Perhaps the most striking examples are found in *Nosema marionis* (1.5-7μ long), *Stempellia mutabilis* (2-6μ long), *S. magna* (12.5-16.5μ long), etc. A somewhat regular dimorphism has been noted by several investigators, while others hold that such a state does not exist.

The spore is covered by a spore membrane and contains a sporoplasm and a polar filament. The spore membrane is fairly refractive, and ordinarily of a uniform thickness. Its outer surface is smooth and structureless. In three species (*Thelohania giardi, Gurleya tetraspora* and *Mrazekia piscicola,* Cépède, 1924), fine longitudinal striæ were noted. Unlike MYXOSPORIDIA, the spores of MICROSPORIA rarely bear appendages, only four species being known to possess any (*Mrazekia caudata, M. brevicauda, M. piscicola* and *Thelohania octospora*). Because of the minuteness of the majority of spores, it is a matter of dispute whether the spore-membrane is composed of two valves, as in myxosporidian spores, or of a single piece. In the great majority of species, it appears to be a single piece, while in a few species the bivalve nature of the membrane has been established. Little is known concerning the chemical nature of the spore membrane. The spore membrane of *Nosema bombycis* and *N. apis* was found to be composed of chitinoid substance.

The structure of the microsporidian spore has been, and still is, disputed, which is without doubt due to the minuteness of the object, to the peculiar nature of the spore membrane, to the present state of optical apparatus and microscopic technique and to the probable dissimilarity of structure among different species.

When viewed in the fresh condition, the microsporidian spore may show either a distinct vacuole or clear space at one end (as in *Nosema cyclopis,* etc.) or two large vacuoles, one at each end (as in the species of *Glugea*). In larger spores, the filament may be visible, but the sporoplasm is indistinct. As to the presence of a polar capsule and the position of the sporoplasm, opinions vary even in those supposed to be one and the same species.

The fundamental characteristic of the microsporidian spore is the presence of a single polar filament (two in DICNIDEA). In a few species with large spores, it is visible in life (as in *Stempellia magna*), but ordinarily the polar filament is only to be noted when extruded from the spore. When extruded, it is conspicuous in that it is extremely fine and long. In an extreme case such as in *Plistophora longifilis*, it reaches a length of 500 microns. The polar filament is of uniform thickness throughout. In genus *Mrazekia,* however, it apparently is made up of two portions: a more or less thick basal rod-like structure ("manubrium") and the other uniformly fine filament. In the other genera, the end with which the filament is attached to the spore is thickened to form a basal granule ("Polkörper," Zwölfer, 1926; "corp polaire," Debaisieux, 1928).

The end from or near which the polar filament becomes extruded is ordinarily known as the anterior end, while the opposite the posterior end. If the spore is pyriform in form, the narrow end is, as a rule, the anterior end.

MATERIAL

The MICROSPORIDIA are typically cytozoic. Recently, Weissenberg (1926) found that *Nosema binucleatum* develops in the non-cellular ground substance of the tunica elastico-muscularis of the mid-gut of *Tipula gigantea* (larva) and this seems to be the only known form which attacks intercellular space of the host's tissue. Up to the present, there have been known two species of MICROSPORIDIA which attack every tissue of the host. They are the well-known *Nosema bombycis* and *N. nonagriæ* parasitic in the larva, pupa and adult of *Nonagria typhæ* (Schwarz, 1929). Aside from these two species, the microsporidian species appear to be confined in a few tissues and the majority of species attack only the cells of a particular tissue.

In the case of a mild infection, no macroscopic changes appear on the host's body. When the infection is very severe, conspicuous changes are to be noted in the host's body. In small aquatic arthropods, the heavily infected animals will easily be detected as suffering from microsporidian infection by an opacity which is characteristic. The pathological changes of the host body due to severe microsporidian infections, which had been known up to 1924, have been summarized elsewhere, to which the reader is referred (Kudo, 1924: 35-40).

As to the sources of material for a laboratory study, larvæ of various genera and species of mosquito may be conveniently obtained. Other dipterous larvæ, as, for example, *Simulium* larvæ, and crustaceans such as *Cyclops* or *Gammarus,* are also excellent material to study. Examination of the gut of a number of honeybees will in most cases result in finding *Nosema apis.* In the localities where the silkworms are raised, one finds, even at the present day, little difficulty in discovering some larvæ infected by *Nosema bombycis,* the most widely studied microsporidian. Among the fish, North American smelt (*Osmerus mordax*) has been found to be a frequent host to *Glugea* sp. (probably *hertwigi*). This microsporidian forms cysts in various parts of the intestine of the host. As to the known MICROSPORIDIA and their hosts, the reader is referred to Kudo (1924).

METHODS

The methods described for MYXOSPORIDIA also hold good on the whole for MICROSPORIDIA. Seen under a low magnification, microsporidian spores appear uniform in size, shape and appearance, are highly refractive and are as a rule heavier than other matter present in the field so that they are always found in the lowermost focal plane. A clear space or vacuole may be found in many species at one pole of the spore, but it cannot be depended upon as a characteristic of a microsporidian spore, since a similar appearance has been recognized in the spores of HAPLOSPORIDIA or in yeast cells. To casual observers, certain PROTOPHYTA may sometimes appear as microsporidian spores. Mercier distinguishes a microsporidian spore from a yeast cell by staining with Ziehl's fuchsin followed by decoloration with a weak sulphuric acid solution. The former then remains bright red, while the latter become colorless. Sturtevant reported that the pollen grains of corn contained oval starch granules similar in size and appearance to the spores of *Nosema apis.* In such a case, an addition of iodine solution would reveal the real nature of the object.

The most important characteristic of the microsporidian spore is the presence of the polar filament which can be extruded comparatively easily. As in the case of MYXOSPORIDIA, various reagents have been proved as capable of causing the filament-extrusion from fresh microsporidian spores (see Kudo, 1924, pp. 22 and 58). It seems, however, that one reagent may be efficient in bringing about the filament-extrusion in certain species, while it may fail completely in other species, which reason remains undetermined. Schwarz (1929) used a concentrated watery solution of methylene-blue for vital staining with two per cent acetic acid, and Zwölfer (1926) successfully used methylene-blue (1 : 1000) and neutral red (1 : 100). Georgévitch (1929) succeeded in causing the filament-extrusion by Schulze's solution which had been used previously by Zwölfer (1926) for staining extruded polar filaments. Although considered to be too violent and consequently the extrusion to be unnatural, the mechanical pressure method is preferable to other methods in many cases when the material is too scanty to test out which reagent would be effective, since it brings out without failure the filament of the microsporidian spore.

Although the extruded polar filaments are noticeable without staining with an ordinary microscope, the darkfield microscope will greatly facilitate their detection. To make permanent preparations, use Löffler's, Fontana's, Giemsa's or Schulze's solution. Addition and spreading of a drop of ink or nigrosin and mounting in Canada balsam when dry, makes excellent preparations (Morgenthaler).

Besides the spore, the schizonts and sporonts at various stages of development may be noted in the fresh hanging-drop preparation. The sporonts are more or less easily noticed especially in polysporoblastic species, since the developing spores are grouped in characteristic manners. These should be studied and measured. The schizonts are not very readily recognized in the fresh condition, because of the smaller size as well as of lack of distinct characteristics.

The presence of the polar filament in the spores suffices one to hold definitely that the spores are those of a microsporidian. Aside from a single genus in which each spore possesses two polar filaments, all the other species of MICROSPORIDIA possess spores with a single filament only. To which family the microsporidian belongs, can be determined by the general form of the spore. In some cases, the generic determination may be possible in the fresh condition. For example, if one sees numerous octosporoblastic or octosporous sporonts together with ovoid or pyriform spores, one is fairly certain that the species belongs to *Thelohania.* But the generic and especially specific determination will be accomplished only after a careful study of section and smear preparations.

It should be noted here that the difference in the length of extruded polar filament possesses little value in taxonomic consideration, since various methods cause different degrees of filament-extrusion.

As to fixatives, those that have been mentioned for MYXOSPORIDIA are commonly and satisfactorily used. Debaisieux (1928) believes that Bouin's mixture is good for the vegetative stages and Schaudinn's fluid for stages in sporogony and the spore, since the latter is too violent and hence destructive to the vegetative form. If the material is abundant, it is wise to fix it with various fixatives.

The sections should on the whole be thinner ($2\text{-}6\mu$) than those for MYXOSPORIDIA and are more important than in the case of the latter, as the developmental stages of the parasite are to be made out with certainty by studying them only.

Stained spores are noticeably smaller than fresh spores, due partly to the shrinking through fixation and partly to the similar refractivity of the spore membrane and Canada balsam. For this reason, one must use care here also as in MYXOSPORIDIA in comparing the dimensions of the spores under observation with those of the spores of known species which have been observed under various circumstances.

Whether preserved spores can extrude their polar filaments or not has not been thoroughly investigated. The writer treated the spores of *Nosema bombycis* with ten and thirty per cent alcohol for sixteen hours, and found that some of the spores extruded their filaments upon addition of perhydrol or application of mechanical pressure.

PROBLEMS

1) *The structure of the spore.* Concerning the structure of the microsporidian spore, a great diversity of opinion prevails. Apparently there are several reasons for this state. In the first place, the microsporidian spore is, as a rule, minute and covered by relatively thick spore membrane which hinders a clear view of its contents. Secondly, various fixing and staining methods do not give uniform results even in one and the same species. On the other hand, the

difference in structure of spores of various forms studied by the same investigator using the same methods would lead one to think that different microsporidian spores are not similarly constructed. Without taking this point into account, investigators have attempted to interpret the structure of microsporidian spores studied by other observers on the basis of their own observations, which has resulted in a confused state of our knowledge, as indicated in the following summary of opinions regarding the subject:

a) A definite polar capsule is present in the spore.

(1) The sporoplasm is girdle-like (ring in cross-section) which surrounds the polar capsule at the middle portion of the spore. Thélohan was the first one to attempt to interpret the structure of a microsporidian spore. He made out, by heating the spores with nitric acid, a pyriform polar capsule which was surrounded by a coat of protoplasm. Mercier (1908) observed that in a mature spore of *Thelohania giardi*, there is a pyriform polar capsule with a coiled polar filament, which occupies about two-thirds of the intrasporal cavity and attaches itself to the narrow end of the spore. The sporoplasm presents a girdle-like form at the central part of the spore, through which the capsule penetrates. Stempell, Schröder, Kudo, Schwarz (1929) and others, agreed on the whole with Mercier. Some authors, for instance Fantham and Porter, considered the vacuole near the anterior end as the polar capsule.

(2) The sporoplasm is located near the posterior end and the polar capsule in the anterior portion of the spore. Léger and Hesse (1907) considered that in the spore of *Nosema bombycis* there was a comparatively large polar capsule near one end and the sporoplasm was located close to the opposite end. These authors found later a similar structure in *Plistophora macrospora* (1916*a*). Similar structure was observed in the spores of *Plistophora elegans* (Auerbach), *Thelohania acuta* (Schröder), *T. varians* (Debaisieux), *Plistophora* (*Glugea*) *danilewski* (Guyénot and Naville), *Nosema apis, N. cyclopis, N. anophelis, Thelohania obesa, Stempellia magna* (Kudo), *Thelohania vandeli* (Poisson, 1924), *Nosema nepæ* (Poisson, 1928), etc.

b) There is no polar capsule; the polar filament occurs exposed to the intrasporal cavity.

(1) The polar filament occupies one end, while the sporoplasm the other. Léger and Hesse (1916) found that in the spores of *Mrazekia*, which are cylindrical in form, the polar filament which

is composed of two parts, a proximal solid portion and the distal portion, occupies a large portion of the intrasporal cavity, while a small rounded sporoplasm is present at the posterior end. An exactly same structure was recognized in *Mrazekia piscicola* (Cépède, 1924), *M. niphargi* and *Toxoglugea mercieri* (Poisson, 1928).

(2) The sporoplasm is present near the middle part of the spore in a girdle-form, and the polar filament is coiled in the posterior space of the spore and connected with the anterior end through the sporoplasm ring. In the spore of *Plistophora longifilis* Schuberg (1910) observed that there was a girdle-like sporoplasm a little toward the anterior end and that the polar filament was coiled directly under the spore membrane mostly in the posterior portion of the intrasporal cavity. A similar structure was recognized in the spores of *Nosema apis* (Fantham and Porter; Trappmann), *N. bombi* (Fantham and Porter), *Glugea anomala, G. hertwigi* (Weissenberg), *Nosema bryozoides, N. glossiphoniæ* (Schröder), *N. bombycis* (Omori), *Plistophora blochmanni* (Zwölfer, 1926), and *Thelohania ephestiæ* (Mattes, 1928).

Thus one finds contradictory views on the structure of even one and the same species of MICROSPORIDIA. For examples, note the views of various authors on *Nosema bombycis, N. apis* and *Plistophora* (*Glugea*) *danilewskyi.*

Judging from the observations made on comparatively large spores, it seems safe to state that there are at least four types of microsporidian spores with regard to their structure. They are:

(1) Spore ovoidal, one polar capsule at each end. The sporoplasm in band-form between the capsules. Example: *Telomyxa glugeiformis* after Léger and Hesse (1910).

(2) Spore cylindrical; no polar capsule; polar filament composed of basal rod-like part and the filament, occupying the greater part of the spore; the sporoplasm at the opposite end. Example: *Mrazekia argoisi* after Léger and Hesse (1916).

(3) Spore pyriform; polar capsule present occupying anterior half or two-thirds of the intrasporal cavity; the sporoplasm at the posterior end. Examples: *Plistophora macrospora* after Léger and Hesse (1916*a*); *Stempellia magna* after Kudo (1921, 1924).

(4) Spore ovoidal; no polar capsule; the polar filament is coiled in the posterior cavity of the spore; the sporoplasm in girdle-like

form near the middle of the spore, through which the filament reaches the anterior end. Example: *Plistophora longifilis* after Schuberg (1910).

It is interesting to note here again, as the writer has done before, that the structural variation in microsporidian spores seems to be delimited by MYXOSPORIDIA on one hand and HAPLOSPORIDIA on the other. In genera *Coccomyxa, Unicapsula* and *Myxobolus* (unicapsulated), one finds a pyriform polar capsule at one end and a sporoplasm at the other, which is exactly similar to the spores of *Plistophora macrospora* or *Stempellia magna*. In *Telomyxa glugeiformis*, the only bicapsular microsporidian, one finds a close structural similarity between it and myxosporidian spores belonging to genera *Myxidium* or *Zschokkella*. On the other hand, forms such as *Octosporea muscæ-domesticæ* or *O. monospora* seem to show somewhat close affinity to HAPLOSPORIDIA.

2) *The nucleus of the sporoplasm.* As to the number of the nuclei present in the sporoplasm of presumably mature spores, observations vary even in one and the same species according to different observers.

A single nucleus was noted in *Nosema bombycis* (Omori), *N. apis* (Kudo), *N. ctenocephali* (Korke), *N. bætis, N. cyclopis, N. anophelis, Thelohania obesa, T. pyriformis* (Kudo), *T. varians* (Debaisieux), *T. corethræ* (Schuberg and Rodriguez), *T. vandeli* (Poisson, 1924), *Plistophora longifilis* (Schuberg).

One or two nuclei were noted in the spores of *Nosema bombycis* (Léger and Hesse), *Thelohania acuta?* (Schröder), *T. legeri* (Kudo), *Toxoglugea mercieri* (Poisson, 1924) and *Plistophora blochmanni* (Zwölfer, 1926).

Two nuclei were noticed in *Nosema bombycis* (Kudo), *N. bryozoides* (Schröder), *N. marionis* (Georgévitch), *N. apis* (Fantham and Porter, Massen, Trappmann), *N. bombi* (Fantham and Porter), *N. glossiphoniæ* (Schröder), *N. frenzelinæ* (Léger and Duboscq), *N. binucleatum* (Weissenberg, 1926), *N. nepæ* (Poisson, 1928), *N. nonagriæ* (Schwarz, 1929), *Perezia mesnili, P. legeri* (Paillot), *Glugea stephani* (Woodcock), *Plistophora (Glugea) danilewskyi* (Guyénot and Naville), *Thelohania ephestiæ* (Mattes, 1928), *Plistophora macrospora* (Léger and Hesse), *Mrazekia argoisi, M. brevicauda, M. caudata* (Léger and Hesse), *M. mrazeki* (Hesse), *M. piscicola* (Cépède, 1924), *M. nipharg i* (Poisson, 1924), *Octosporea muscæ-domesticæ* (Chatton and

Krempf), *O. simulii* (Debaisieux, 1928) and *Telmyxa glugeiformis* (Léger and Hesse.)

Four nuclei were reported to occur in *Glugea anomala, Nosema bombycis* (Stempell) and *Thelohania giardi* (Mercier). The above tabulation would seem not to support Debaisieux's (1928) recent generalization, which makes a part of an excellent paper, that the spore of the species which possess unisporoblastic sporonts, contains a binucleate sporoplasm, while that of the species which possess polysporoblastic sporonts, a uninucleate sporoplasm.

3) *The structure and function of the polar filament.* The polar filament of the microsporidian spore was first discovered by Thélohan (1892) in *Glugea anomala.* Although it had frequently been compared with the tubular thread of the nematocyst of the cœlenterates, it was Stempell (1909) who supposed that it was probably tubular in structure. Strickland and Morgenthaler agreed with Stempell. Oshima (1927) repeated the writer's observation on the filament-extrusion of the spores of *Nosema bombycis* (Kudo, 1918) by means of hydrogen peroxide and claimed to have seen evidences which led him to consider the filament is tubular by careful examination under a darkfield microscope.

As to the function of the polar filament, it is generally agreed that it serves as a temporary anchoring apparatus of the spore in the gut lumen of a new host. Korke (1916) thought further that it may serve to conduct the amœbula to a distant part of the host tissue. Ishiwata (1917) noted a round knob which seemed highly adhesive at the end of the extruded filament of *Nosema* sp. Morgenthaler (1922) observed often in *Nosema apis* a fluid mass at the end of the extruded filament. In *Stempellia magna,* Kudo (1924) noted occasionally a thick point at the end of the filament and considered it as the material of the filament which was spread out by the breaking of the structure. Zwölfer (1926) recognized the viscosity of the surface of the extruded filament of *Plistophora blochmanni.* These observations strengthen the view that the filament serves for bringing the spore close to the host tissue. According to the same observer trypsin dissolved the filament completely after twenty-four hours. Oshima (1927) noticed that the extruded polar filaments of *Nosema bombycis* were digested by the digestive fluid of the silkworm larva in two to five seconds and considered that the filaments penetrate through the epithelial cells of the alimentary canal of the host larva and discharge their contents, which "weaken the activity of the epithelial cell."

As to the mechanism of the filament-extrusion, Stempell (1909) maintained that the sporoplasm increases its volume by absorption of water through the spore membrane and the pressure causes the extrusion of the filament. Kudo (1918) held that probably "a physical force, comparable to that produced by pressure, develops within the spore and expels the polar filament. This force may be none other than the gas evolved through the decomposition of hydrogen peroxide by the peroxydase contained within the spores." Morgenthaler (1922) holds that since the water is capable of causing the extrusion of the filament, there may be in the polar capsule a substance which increases its volume by absorbing water and this results in the filament-extrusion.

4) *The spore-membrane.* In the majority of MICROSPORIDIA, the spore membrane seems to be of a single piece. A longitudinal line which was recognized as the sutural line of the two valves of the spore-membrane was noted, however, in *Glugea anomala, Thelohania giardi* (Thélohan), *Plistophora simuli* (Lutz and Splendore), *P.* sp. (Mercier), *P. blochmanni* (?) (Georgévitch, 1929) and *Thelohania opacita* (Kudo). From this brief summary it would seem that the majority of microsporidian spores possess a spore-membrane of a single piece, while in a few species, the spore-membrane is apparently composed of two valves.

5) *The dimorphism of the spore.* As was stated briefly in the section on the spore, a number of observers noted a dimorphism in the microsporidian spore. Léger (1897) noticed two types of spores in *Thelohania varians* with regard to the size, the macrospore and the microspore, the former grouped in variable numbers in spherical masses, while the latter regularly in groups of eight, surrounded by a membrane.

a) Cases of dimorphism. The following species are known to show a more or less regular dimorphism of the spores: *Plistophora mirandellæ* (Vaney and Conte), *Thelohania janus* (Hesse), *T. opacita* (Kudo), *Gurleya richardi* (Cépède), *Plistophora longifilis* (Schuberg), *P. bufonis* (Guyénot and Ponse, 1926) and *P. blochmanni* (?) (Georgévitch, 1929).

b) Is there any structural difference between the macrospore and microspore? Hesse (1903) found in *Thelohania janus* that the macrospore did not possess the polar filament which was present in the microspore. Cépède (1911) and Strickland (1913) made similar observations in *Gurleya richardi* and *Thelohania fibrata* respectively. Guyénot and Ponse (1926) expressed their opinion that the macrospore and microspore may not react to chemical reagents in the same manner. On the other hand, Schuberg (1910) observed that the two types of spore of *Plistophora longifilis* were similar in structure. The writer agreed with this view.

c) How are the two types of the spore produced? Hesse noted in *Thelohania janus,* octosporous and tetrasporous sporonts gave rise to the microspores and macrospores respectively. The writer saw a similar condition in *Thelohania opacita* (Kudo, 1922). Guyénot and Ponse (1926) noted in *Plistophora bufonis* that there were two types of schizogony which resulted in producing some sporonts in which four, eight or about sixteen macrospores become developed and others in which thirty-two to about sixty-four microspores become formed. Thus it seems that the so-called dimorphism of the spores of polysporoblastic species is due to the number of sporoblasts which develop in the sporont.

d) What is the significance of the dimorphism? Vaney and Conte (1901) supposed that the macrospore serves for propagation of the parasite within the host body, while the microspore for infection of new host individuals. Guyénot and Naville (1922*a*) who noted an ambiguous dimorphism (macrospore 4μ long and microspore 3μ long) in *Plistophora* (*Glugea*) *danilewskyi,* were inclined to suppose that an anisogamy precedes the formation of macrospores, while the microspores are produced "asexually." In *P. bufonis,* Guyénot and Ponse (1926) saw three chromatin granules ("haploid") in the sporont which is destined to produce the microspores, without however succeeding in finding the number of chromatin granules in the nuclear division of macrosporoblastic sporonts.

The writer held, and still holds, that in at least *Thelohania opacita* the large and small spores are simply due to development of four and eight spores in a sporont, and does not find any significance such as is briefly considered here.

6) *Artificial cultivation.* Attempts to cultivate MICROSPORIDIA have so far failed. Even the actual emergence of the sporoplasm has been observed in few cases. Investigation with respect to artificial cultivation of the microsporidian is highly desirable.

7) *Problems concerning infection through the digestive tract.* The fact that the silkworm larva becomes infected by *Nosema bombycis* through ingestion of spores has been repeatedly proved. Fantham and Porter were able to infect the honey-bee by feeding it on honey to which spores of *Nosema apis* were added. Weissenberg (1913, 1921) succeeded in obtaining positive experimental infections with *Glugea anomala.* The writer also succeeded in infecting the larvæ of *Culex territans* with the spores of *Stempellia magna* (Kudo, 1925).

On the other hand, a number of investigators obtained negative results by carrying out experimental infections. Henneguy and Thélohan (1892) failed to obtain positive results by feeding the host crustaceans with *Thelohania giardi* and *T. octospora.* Henneguy thought that the infection probably does not take place through the digestive tract in view of the fact that lesions are found at first at places remote from the alimentary canal. A somewhat similar case was recently noted by Mattes (1928). Goodrich (1920) kept under observation a prawn infected by *T. octospora* in an aquarium with normal prawns and other crustaceans, without finding any infection among any of these crustaceans. Wenyon (1926) tried without success artificial infection of *Thelohania* sp., which had been discovered by MacGregor in the larvæ of *Aëdes nemorosus* and remarked that it seemed evident that infection depends on certain conditions not at present known.

Zwölfer (1926) likewise failed in bringing about infection in *Gammarus pulex* by feeding with spores of *Plistophora blochmanni* taken from freshly killed host animal, although he noted numerous empty spores in the host's gut lumen fifteen hours after feeding and also numerous binucleate amœbulæ which appeared to have emerged from the spores. No stages of the parasite were, however, found in the body cavity or muscles, although some experimental animals were kept as long as six weeks. Zwölfer could not explain this failure, since the spores were apparently fully mature as seen from the emergence of the amœbulæ. Mattes (1928) also obtained negative results by feeding normal host larvæ (*Ephestia kuehniella*) with *Thelohania ephestiæ;* the spores recovered from the fecal matter or the gut of the experimental larvæ did not show any noticeable changes.

Why these failures? Does a spore have to pass through a long resting period in order to become infective? Zwölfer thinks this is improbable. Mattes thought of this and also wondered if the spores had first to pass through certain carriers as was suggested by some of the former workers on MICROSPORIDIA. The determination of factors which favor infection rests upon the work of future investigators.

8) *Problems concerning other modes of infection.* Besides infection through the alimentary canal, infection of ovarian eggs which occurs in numerous species (Kudo, 1924), would result in the hereditary infection of the next generation. This has clearly been established in *Nosema bombycis* since Pasteur's investigation. Marchoux, Salimbeni and Simond (1903) observed that *Plistophora stegomyiæ* is transmitted through ovarian eggs of the host, *Aëdes ægypti.* Chatton and Krempf (1911) considered that since *Octosporea muscæ-domesticæ* is found in the yolk of ova of the host fly, the infection may be transmitted through the germ cells. Schwarz (1929) has recently shown with her beautiful study that the ovarian egg of the imago of *Nonagria typhæ* was found infected by the vegetative stage of *Nosema nonagriæ,* which are massed near the central portion of the infected egg. It seems possible that here also the young embryo may become infected in a way similar to that of *Bombyx mori.*

In the opinion of those who failed to obtain positive results by experimental feeding with proper spores, there must be other modes of infection. One of these is the direct entrance of mature spores through the wounds. In the case of the silkworm, one frequently finds wounds which develop at the time of molting on the body surface, particularly of the prolegs. It was often suggested that the spore may gain entrance to the body through such wounds. It is, however, improbable that this occurs, since the spore is immobile and so far it is not known to germinate in the body-fluid of the host larva, although Sasaki (1897) reported that he saw the emergence of the sporoplasm from the spore which had been placed in the "blood" of silkworm larvæ. In inoculation experiments which, certain workers claimed, were successful in bringing about positive infection, there is always a great possibility that the spores gained entrance through the alimentary canal. As he failed in the experimental infection of *Ephestia* larvæ by feeding the spores of *Thelohania ephestiæ,* Mattes (1928) suspected that the stings of the ichneumon-flies did not cause new infection, but failed to obtain results by a few experiments. It seems worth while to undertake a careful infection experiment by inoculation to establish a definite fact.

9) *Viability of the microsporidian spore.* As to how long the microsporidian spore would live under various circumstances, a number of experiments have been carried on by several investigators of *Nosema bombycis* and of *N. apis*. In both cases, the results were obtained by feeding healthy larvæ or bees with the treated spores mixed with the food material. Spores of *Nosema bombycis* contained in dried moths may retain their life for over a year if kept at room temperature, while fermentation in the fecal matter of the silkworm larvæ destroys the spores within about two months. White (1919) carried on a number of experiments with *Nosema apis* and obtained interesting results.

Viability of the microsporidian spores of the species which attack aquatic animals has not been studied at all. In this connection, mention must be made of the interesting observation of Goodrich (1928). A number of glass bowls in which *Gammarus pulex* suffering from infection of *Thelohania mülleri* were kept, were washed (but not sterilized) and later used for rearing of *Gammarus chevreuxi* which had been bred in the laboratory for at least three or four generations. Some of the latter were found to have become infected by the microsporidian, which fact could only be explained by assuming that the spores of *Thelohania mülleri* which had been formed in *Gammarus pulex* survived for at least six months and most likely thirteen months or more to become the source of infection.

10) *Problems concerning the emergence of the sporoplasm as the amœbula.* Although there had been a number of attempts to see the changes which the spore undergoes when it is introduced into the body of a new host animal, it was Stempell (1909) who first succeeded in making out a more or less complete life-history of the well-known microsporidian, *Nosema bombycis*. According to this investigator, the mature spore has four nuclei in its sporoplasm. When the spore enters the host's gut, the polar filament is extruded under the action of the digestive fluid and later becomes detached completely from the spore. Through this foramen, the sporoplasm which leaves two of its four nuclei behind, creeps out of the spore membrane to become an amœbula in which two nuclei fuse into one. This uninucleate amœbula ("planont") multiplies actively by a binary fission or budding. It is capable of amœboid

movements (not actually seen) in the lumen of the alimentary canal, and the hemocœle, and finally enters a host cell to begin intracellular development. By mixing fresh spores of *Nosema bombycis* with a drop of the digestive fluid of healthy larvæ on a slide, and keeping it at the room temperature for twenty-four hours, the writer noted the sporoplasm in some spores moved toward the anterior end as a round or irregular mass and caused the extrusion of the filament (Kudo, 1916). Further observation could not be carried on because of the bacterial growth. Through infection experiments with the same microsporidian, it was found that the oblong or triangular amœbula is binucleate and capable of amœboid movements judging by its irregular form. This stage seemed to give rise to a uninucleate stage. The so-called planont stage of Stempell was not observed.

An infection experiment with *Stempellia magna,* led the writer to state that the spores extruded the filaments in the posterior region of the midgut, and the uninucleate amœbulæ creep out. They are capable of amœboid movements and penetrate through the gut epithelium and surrounding tissues (manner unknown). They finally enter the fat body surrounding the gut or adjacent tracheæ.

Thus it is to be noted, from these small number of incomplete observations on experimental infection, that when the microsporidian spore gains entrance with the food into the midgut of a suitable host, it extrudes its polar filament under the action of the digestive fluid and the sporoplasm emerges through the opening in the spore membrane. Although it is agreed in general that the sporoplasm escapes through the foramen which was made by the detachment of the extruded filament, certain authors report that the emergence of the sporoplasm may take place at other places (Sasaki, Paillot, Guyénot and Naville, Zwölfer). Georgévitch's (1929) hypothetical figure is apparently based upon his view that the spore membrane is of bivalve shell and is unfortunately not based upon actual observation. It is regrettable that there is no experimental observation on the emergence of the sporoplasm in the spores in which the sutural line was noted.

Stempell's view about the planont stage needs further confirmation.

11) *Problems in schizogony.* When an amœbula enters a host cell, it becomes a schizont ("meront" of Stempell). How an

amœbula makes its way through the host's cell wall is not understood. As a rule, the cytoplasm of a schizont takes stain more deeply than that of a sporont. In the majority of species, the youngest schizont is uninucleate. The schizonts multiply by simple and repeated binary and multiple fissions and increase in number within the cell. The nuclear division appears to be amitotic, though presenting various aspects among different species. In a few species such as *Thelohania mœnadis, T. giardi, Toxoglugea mercieri* (Poisson, 1924), the division is said to be mitotic or promitotic. Through multiplication, a large number of uninucleate schizonts are produced, and ultimately each is considered as giving rise to a uninucleate sporont. This change was reported to occur in *Nosema bombycis* (Stempell, Kudo), *N. apis, N. bombi* (Fantham and Porter), *N. anophelis* (Kudo), *N. marionis* (Stempell), *N. frenzelinæ* (Léger and Duboscq), *Thelohania chætogastris* (Schröder), etc.

In other species, the youngest schizonts appear to be uninucleate, and multiply by binary or multiple fission in number. From these, binucleate schizonts ("diplocarya" of Debaisieux) are formed, as a result of the nuclear division, without being accompanied by cytoplasmic division. This change was noted in *Thelohania multispora, T. fibrata, T. bracteata* (Debaisieux and Gastaldi), *T. chironomi, Plistophora simulii* (Debaisieux, 1928), *Thelohania legeri, Stempellia* (Kudo). These binucleate schizonts seem to multiply in number by division. Finally the two nuclei fuse and a uninucleate sporont is produced.

In a number of species, the young schizonts appear to contain two nuclei. This was reported in *Thelohania corethræ* (Schuberg and Rodriguez), *Perezia mesnili, P.? legeri* (Paillot), *Nosema binucleatum* (Weissenberg, 1926), and *N. nonagriæ* (Schwarz, 1929). These binucleate schizonts multiply by binary or multiple fission, which results in the increase in number. Finally each schizont gives rise to a binucleate sporont.

According to Mattes (1928), the amœbula of *Thelohania ephestiæ* is first binucleate and multiplies in number by a binary fission. Uninucleate schizonts are formed from these through nuclear fusion, which also multiply by a binary or multiple division. Later binucleated schizonts develop from them. The fusion of the two nuclei in these binucleate schizonts results in the production of uninucleate sporonts.

Any attempts to generalize schizogonic changes as reported by various investigators will at present only increase the confusion which already exists. Careful cytological studies of species in which large schizonts occur are greatly desired.

12) *Problems in sporogony.* As was stated above, schizogony terminates when each schizont transforms itself into a sporont, with or without nuclear fusion. In two cases (*Thelohania giardi* after Mercier; *Plistophora* (*Glugea*) *danilewskyi* after Guyénot and Naville), however, the sporont is said to be produced by a union of two gametes.

Each sporont gives rise to one, two, four, eight, sixteen or more sporoblasts according to various genera (Kudo, 1924: 65-69). The nuclear division which takes place in the course of sporoblast formation appears to be amitotic in numerous species. In some species of *Thelohania,* typical mitotic figures were observed. It appears to be seen clearly in the species of this genus parasitic in the larvæ of dipterous insects (Debaisieux, Nöller, Kudo). In *Thelohania legeri,* the writer saw in the first nuclear division of the sporont that the nucleus undergoes a spireme stage, followed by the appearance of chromosomes ("pseudochromosomes" of Debaisieux, 1928) and spindle fibers, and the disappearance of the nuclear membrane. The number of the chromosomes could not be made out distinctly, but it appeared to vary from six to ten and in anàphase eight were fairly regularly made out. In *T. multispora,* Debaisieux and Gastaldi noted eight chromosomes at the equatorial plane, and eight or nine at telophase, and in a later paper, Debaisieux (1928) stated that at each pole in telophase he counted eight or nine chromosomes in the first and five in both second and third nuclear divisions. The spindle fibers were very distinct.

As to the development of the sporoblast into a spore, there are various observations.

a) Léger and Hesse (1907) remarked briefly that the spore of *Nosema bombycis* possesses two cells for the spore membrane, a polar capsule with a small nucleus and a mono- or binucleated sporoplasm. Stempell (1909) in the same species and Mercier (1909) in *Thelohania giardi* observed that the nucleus of the sporoblast divides into five nuclei, of which two form the spore membrane, one the polar capsule and the filament and two the sporoplasm. The same view was expressed by Fantham and

Porter for *Nosema apis* and *N. bombi,* Stempell for *N. marionis* and *Thelohania mülleri,* Kudo for *Nosema bombycis* and recently by Poisson for *Toxoglugea mercieri* (1924) and *Nosema nepæ* (1928).

b) Schuberg (1910) noted in *Plistophora longifilis* that the uninucleate sporoblast transformed itself into a spore without division of its nucleus. Similar observations were made by Weissenberg (1913) in *Glugea anomala* and *G. hertwigi,* Debaisieux in *Thelohania varians, Glugea mülleri, G. danilewskyi* (1919) and *Plistophora simulii* forms (1928).

In *Stempellia magna,* the writer noted the sporoblast is uninucleate and transforms itself into a spore. There seems to be no nuclear division in this process, but numerous granules were noticed in the developing polar capsule.

c) In case the sporoblast contains two nuclei, the transformation of the sporoblast into a spore appears to go on without nuclear division or fusion. This state has been observed by Paillot (1918) in *Perezia mesnili* and *P. legeri,* Weissenberg (1926) in *Nosema binucleatum,* Debaisieux (1928) in *Octosporea simulii* and Schwarz (1929) in *Nosema nonagriæ.*

13) *Problems of the hypertrophy of the infected host cell.* Except a single species, *Nosema binucleatum,* which was excellently studied by Weissenberg (1926) and which develops in the intercellular space of the host larva, all MICROSPORIDIA are cytozoic parasites. Not infrequently the infected host cell becomes highly enlarged, due without doubt to the stimulation and increase in numbers of the parasite through multiplication. This has been shown abundantly in the case of *Nosema bombycis, Thelohania legeri, T. opacita, Stempellia magna, T. tipulæ* (Weissenberg, 1926), etc.

On the other hand, there are a number of cases in which the host cell becomes enlarged even though there are only a few parasitic bodies within it. The writer noted this in *Nosema anophelis.* Debaisieux (1928) observed also a similar state in *Octosporea simulii* and remarked that in such a case the cause of hypertrophy is not due to mechanical action of the parasite, but to certain action which produces the abnormal enlargement of the host cell.

Still further, there are a number of cases in which no noticeable hypertrophy of the host cell occurs, as was reported by Zwölfer

(1926), Schwarz (1929), and others. The solution of this problem depends upon future investigations.

14) *Problems of the hypertrophy of the host cell nucleus.* The MICROSPORIDIA have been considered to confine themselves to the cytoplasm of the host cell. As a rule, the host cell nucleus becomes highly hypertrophied and modified in structure. Typical examples are *Plistophora longifilis, Gurleya francottei, Mrazekia stricta, Stempellia magna, Nosema bœtis, N. nepæ,* etc.

Recently Guyénot and Ponse (1926) noted development of *Plistophora bufonis* in the nucleus of the host's follicular cells. Schwarz (1929) gives a good account as to how the nuclei of adipose tissue and muscle cells of the host larva are attacked by *Nosema nonagriæ* and are completely destroyed.

How is the host cell nucleus hypertrophied? If it is due to certain influences of the microsporidian, as has been considered the case, what is its nature? Of these we have no knowledge at present.

15) *Problems of phagocytosis and other reactions on the part of the host against microsporidian parasites.* A number of cases of phagocytosis were reported to occur (Kudo, 1924). Recently this phenomenon has been observed further in *Nosema binucleatum* (Weissenberg, 1926) and *Thelohania ephestiæ* (Mattes, 1928). Zwölfer (1926) found that when the muscle bundles of *Gammarus pulex* are completely destroyed by *Plistophora blochmanni,* the whole structure becomes surrounded by the host's connective tissue which seems to secrete a substance which cements the microsporidian spores and the whole assumes a dark brownish color. Goodrich (1928) also noted similar conditions in *Gammarus* infected by *Nosema* sp. and *Thelohania mülleri* (?), and observed that the dark brown coloration of the parasitic masses which replaced the host tissue completely was due to the secretion of chitinoid substance by the phagocytes which attacked the peripheral portion of the parasitic mass. Poisson (1928) noted small white or brown "cysts" of *Nosema nepæ* in the gut wall of *Nepa cinerea,* its host. This microsporidian attacks primarily the adipose tissue. In the brown "cyst," a number of spores are encased within an envelope of connective tissue cells and leucocytes. The spores are more or less degenerated and the entire mass is covered by a pigment zone which was considered as degeneration product of the connective tissue cells and leucocytes.

16) *Problems of autoinfection.* As to whether the spores can germinate in the same host animal in which they were produced, there is no precise experimental evidence. Zander and Fantham and Porter held that autoinfection of *Nosema apis* occurs in the infected honey-bee. On the other hand, Trappmann (1926) considers that the spores which were set free by the disintegration of extruded infected gut cell are not mature and do not germinate in the same host. With regard to *Nosema bombycis*, Kudo (1916) held that autoinfection through spore takes place, while Stempell (1909) maintained a contrary view. Paillot (1918), Mattes (1928) and others seem to consider that autoinfection probably takes place in the forms they studied.

17) *Specific host-parasite relation.* We possess a rather meager information on this problem, a summary of which will be found in Kudo (1924).

18) *Problems concerning the seasonal occurrence.* A few investigators only were fortunate enough to continue observations throughout the year and at present data are too inadequate to draw any conclusions (Kudo, 1924).

In the bibliography, references which appear in Kudo (1924) are omitted.

CHAPTER XXXIV

MALARIA IN GENERAL

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INTRODUCTION

Malaria is the most important disease of tropical and subtropical countries. The parasites of malaria and the effects of the parasite on the host have been studied intensively by large numbers of well-trained zoölogists, physicians and public health officers ever since the causative organism was discovered by Laveran in 1880. Nevertheless there are many problems that still remain unsolved and many results that need confirmation. Some of these problems and methods for their study are presented in Chapters XXXV to XXXIX inclusive.

The malarial organisms are blood-inhabiting protozoa belonging to the family PLASMODIDÆ. This family contains a single genus *Plasmodium*. Three species occur in man, *Plasmodium vivax,* the organism of tertian malaria, *Plasmodium malariæ* of quartan malaria and *Plasmodium falciparum* of estivo-autumnal or malignant tertian malaria. Malarial parasites have been described from monkeys. They have been reported from the chimpanzee, gorilla, orang-utan, baboon and over a dozen other species of Old World and New World monkeys. At least eight distinct species have been described from these animals but how many, if any, of these are "good" species is still to be determined. Certain of these parasites in monkeys resemble so closely the three species that occur in man that they are morphologically indistinguishable. Reichenow (1917, 1920) has described parasites in chimpanzees and gorillas in the Cameroons that he believes to be the same as the human species. Similar results were obtained by Blacklock and Adler

(1922) from studies of chimpanzees in West Africa. Malarial infections in monkeys may be acute or chronic as in man. Quinine appears to be effective against them. Efforts to infect mosquitoes by feeding them on malarious monkeys have thus far failed.

A number of cross-infection experiments have been attempted to test the host-parasite specificity of the malarial organisms of monkeys and man. Halberstädter and Prowazek (1907), were able to transfer *P. pitheci* from orang to orang but not to lower monkeys, and *P. inui* from one *Macacus* monkey to another but not to orangs. Mayer (1908) reports successful inoculations of *P. cynomolgi* into *Macacus cynomolgus, M. rhesus* and a *Cercopithecus* monkey. Leger and Bouilliez (1912) were able to infect four species of *Macacus,* three species of *Cercopithecus,* and *Papio anubis* with *P. inui,* but failed to infect *C. fuliginosus* and two chimpanzees. Efforts to infect man with blood from monkeys have not succeeded; Gonder and Rodenwaldt (1910) attempted to parasitize two human beings with *P. kochi,* and Blacklock and Adler (1922) failed to infect man by subcutaneous and intravenous injections of malarious blood from chimpanzees. Mesnil and Roubaud (1917, 1920) claim to have infected a chimpanzee with human blood containing *P. vivax,* but attempts by other investigators to transfer infections from man to monkeys have failed (for example, Blacklock and Adler, 1924). It is obvious from all this that we do not know at present whether the malarial parasites of monkeys and man belong to the same species or not. Further investigations are desirable.

Malarial parasites have been recorded from other mammals including buffaloes in India, duikers in Nyasaland, goats in Angola and dogs and horses in Colombo. Among the smaller mammals, bats seem to be particularly susceptible to malaria. A number of investigators have described several species from these animals. Other small mammals in which malarial parasites have been discovered are the jumping rat in the Belgian Congo, squirrels in Asia and zorillas in Senegal.

The most easily obtainable of all plasmodia in regions where human malaria does not exist are the species that occur in birds. According to Huff (1927) infections have been noted in at least seventy-nine species of birds. These belong to at least fifteen families included in the orders ANSERES, PALUDICOLÆ, COLUMBÆ, RAPTORES, PICI, PASSERES, and others. More species of the order

PASSERES have been found with infections than those of any other order, and members of the family FRINGILLIDÆ appear to be particularly susceptible. The parasites now being used in the laboratories in the United States were obtained from English sparrows by Whitmore in New York in August, 1913, by Hartman in Baltimore in October, 1924, and by Huff in Hampton, Virginia, in September, 1926. Problems and methods of research with these organisms are discussed in Chapters XXXVII to XXXIX inclusive.

Cold-blooded animals are also infected with malarial parasites, particularly lizards. They have been reported from lizards of the southern Sudan and Brazil.

So far as we know the malarial parasites of both man and lower animals are transmitted from one vertebrate host to another by mosquitoes. A considerable body of information exists regarding the mosquito vectors of human malaria and a little is known regarding the transmitting agents of bird malaria (Huff, 1927) but our information concerning the transmission of the other species is very meager.

PROBLEMS IN HUMAN MALARIA

As noted above there are still many problems in human malaria awaiting solution and many of the results already obtained need confirmation. The following list presents in abbreviated form some of these problems. Inasmuch as the life-cycle of the bird malarial parasites and the effects of these parasites on their avian hosts are remarkably similar to those of the human species many of the problems suggested can be attacked with the aid of bird malaria. This is now being done in the writer's laboratory where work has been in progress for ten years and in other laboratories both in this country and abroad.

a. Life-cycle of malaria parasites in the vertebrate host.

(1) Where are the parasites located in the body at various stages in the course of the infection and in what stages in their life-cycles? The distribution of the stages is known to differ in *P. falciparum* from that of the other two species. Why do specimens of *P. falciparum* clog up the capillaries (Bass, 1920)? Why does schizogony of *P. falciparum* take place in the internal organs instead of in the peripheral blood as in the other two species?

What factors bring about these differences in distribution, etc., and what are their relations to virulence, latency, relapse, etc.?

(2) Is the malarial parasite inside or outside of the red cell? In all stages? Do the parasites migrate from one cell to another? (Lawson, 1920; Ratcliffe, 1927.)

(3) Is there a filterable stage in the life-cycle, especially during the period between relapses when no parasites can be found in the peripheral blood? (Boyd, 1925.)

(4) When and where does the change take place from schizonts to gametocytes? Factors? Does the sex of the host have any influence on the production and sex of the gametocytes?

(5) Does treatment with quinine increase the number of gametocytes? (Clark, 1928.)

b. Life-cycle of malaria parasites in mosquitoes.

(1) In nature, what is the influence of temperature, moisture, latitude and altitude on sporogony in the mosquito? On the length of different stages? On the numbers of oöcysts? On the numbers and distribution of sporozoites? Does the environment of the mosquito or its larva or pupa have any effect on its infectivity? Humidity, temperature, salinity of water, food, altitude?

(2) What effects do starvation, various diets, etc., have on stages in the life-cycle in mosquitoes? Duration of stages? Numbers? Length of infectivity?

(3) Are stages in sporogony as worked out by Grassi (1901) correct?

(4) Are different species of mosquitoes responsible for the appearance of *P. vivax* early and *P. falciparum* later in the season? Are mosquitoes responsible for the local appearance of malaria? *Plasmodium malariæ* may be abundant in a spot surrounded by *P. vivax* and *P. falciparum* but free from *P. malariæ*.

(5) Do mosquitoes suffer from infection? Do non-infected mosquitoes live longer than infected mosquitoes?

(6) What effects do hibernation and estivation have on sporogony?

(7) How many gametocytes are necessary to infect mosquitoes? (Darling, 1910.)

(8) How many oöcysts are necessary to produce an infective mosquito?

(9) There are various degrees of susceptibility among mosquito hosts. Why is there not infection in all hospitable hosts?

c. Relapse in malaria.

(1) Parthenogenesis. What is the status of this phenomenon and its relation to relapse? (Samsonoff, 1925.)

(2) Do old red cells become immune? Are young cells more frequently parasitized? Would relapse result from the rapid increase of young cells?

(3) Is relapse due to changes in the blood plasma which make it easier for merozoites to parasitize cells, or to changes in the phagocytic power of leucocytes?

(4) What effect has the administration of quinine on relapse?

(5) What factors are responsible for latency in malaria?

(6) Does the sugar content of the blood have an influence on relapse? (See Chapter XXXIX.)

(7) Is there a resistant stage or unknown stage of the parasite that brings on relapses?

d. Species.

(1) Is there a *tenue* stage in the life-cycle of *P. falciparum?* (Russell, 1928.)

(2) Are the species in man and primates the same? (Hegner, 1928.)

(3) How many human species are there?

(4) Are there two species or subspecies of *P. falciparum?* (Craig, 1921; Darling, 1921.)

(5) If a mosquito is fed on a mixed infection will it transmit a mixed infection? If so, does this prove the plurality of species?

(6) Is virulence related to different races of parasites?

e. Relation of malaria parasites to the resistance of the host and to blood reactions?

(1) Do malaria patients acquire immunity?

(2) Can a complement-fixation test be devised in malaria with antigens prepared from cultures or organs? A precipitin test? (Taliaferro and Taliaferro, 1928.)

(3) What is the relation between the number of parasites in the blood and clinical symptoms?

(4) What factors are responsible for clinical symptoms?

(5) Does dengue give immunity?

(6) What is responsible for the pathogenicity of *P. falciparum?*

(7) How do provocatives bring about increases in the number of parasites in the blood?

Some idea of the variety of malaria problems available for study and the amount of work being done on this subject can be obtained by reviewing the papers abstracted in the Tropical Diseases Bulletin. For example, in a single number (Vol. 26, No. 1, Jan., 1929) there are listed about 125 papers. These deal with almost every problem mentioned above and with many others. Some of them are as follows: factors influencing the geographical distribution of malaria, the relation between spleen and parasite rates, species of mosquitoes as vectors of malarial organisms, the validity of *Plasmodium tenue,* the early appearance of gametocytes during infections, the loss of gametocytes in strains used for therapeutic purposes, the relative numbers of gametocytes before and after treatment with quinine, the relation between the number of gametocytes and the number of oöcysts, blood destruction in malaria, parthenogenesis of malaria parasites, the incubation period in malaria, the effects of the sugar content of the blood on relapse, the significance of blood groups, relations of malaria to other diseases, the effects of quinine, plasmochin and other therapeutic agents, leucocyte formula, precipitin test, transmission experiments with human malaria to lower animals, origin of malarial pigment, methods of treatment, congenital malaria, control of malaria in various localities and under various conditions, and factors involved in a malarial attack. It is safe to state that more work is being done on malaria and its causative organisms than on any other disease due to animal parasites.

CHAPTER XXXV

METHOD OF PREPARING AND EXAMINING THICK-FILMS FOR THE DIAGNOSIS OF MALARIA

By

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Slides should be well cleaned. It is a good plan to wipe slides cleaned in the ordinary manner with an alcohol-wet cloth to free them from grease. Old slides are usable if not fogged or scratched If any dust gets on the slides in carrying them to the field, brush each one on a clean cloth just before using.

Select slides of a proper length to fit the slide box, and sufficiently thick to fit the mechanical stage of the microscope.

Collecting blood specimens. It is essential that the blood be free from dirt coming from the skin, from dust or other débris. Clean the skin with alcohol and gauze. Dry. Prick the skin deeply enough to allow the blood to well up in a large drop. Touch the slide to the upper part of the drop, avoiding as far as possible the blood which has been in immediate contact with the skin. In any case, avoid rubbing the skin with the flat of the slide or scraping it with the edge. Bacteria or other débris, which confuse the search for parasites in the thick-film, usually come from the dirt which is carried by the blood from the surface of the skin; and cleaning with alcohol and gauze does not always wholly free the skin from such débris.

It is possible to get fair specimens from a dry skin without any preliminary cleaning if one takes the blood from the top of the drop only, but it is better to clean the skin as an additional precaution. We usually take the blood from the upper surface of the middle finger, pricking the skin about one-half centimeter below the base of the nail.

One can spread the blood sufficiently by dragging the drop on the surface of the slide; or one can spread the drop with the sticking needle or corner of the labeled end of the next slide. Put on a large drop, three or four times as much as one would use for a thin film, and spread it over an area of from one to one and one-half centimeters in diameter. It does not matter if the blood is thicker in some parts of the film, and a good deal of latitude is permissible in the amount of blood used; but one should avoid the not uncommon error of using a very small amount of blood and then spreading it so much that it becomes the equivalent of a thin film. If the slide is subsequently to be stained in the upright position, place the thick drop with its lower margin about one-half centimeter from the end of the slide, else a good deal of stain will be required to cover the specimens.

Labels may be written with a wax pencil on the unused end of the slide. It is often convenient to number a batch of slides beforehand. Each batch should be marked with a distinguishing letter or other symbol as well as numbered.

As soon as the blood is spread, place the slide in a box where it will be protected from dust. Keep the box upright until the blood has dried enough so that it will not run. While the box is being filled it may be fastened with a strong rubber band to a block or other convenient support in order to prevent accidental tipping. (Fig. 20, A). Such a support may be made of two blocks of soft wood, fastened to each other at a right angle, the upright block being three and one-half inches wide, one inch thick, and about six inches long. The horizontal block should be three and one-half inches wide, two inches thick and about four inches long.

Drying films. Thick films should be dried enough to make them adhere during the staining, but too much drying will prevent a clear staining of the parasites.

In ordinary summer weather preparations will dry enough to stick well if kept overnight in boxes with closed lids. Or, the lids may be removed and the slides dried from one to one and one-half hours in an incubator. When preparations are to be sent some distance by mail before staining, or when it is impractical for any reason to stain soon after taking them, they may be protected from overdrying for some days by wrapping them in paraffined paper. In any case, they must be protected from flies and cockroaches, which will eat dried films, and from dust. The temperature and dryness of a room, as well as that of an incubator, vary so much in different laboratories that it is impossible to give precise rules for drying, but a very little experience will guide.

Staining. It is essential to use a good quality of Giemsa stain. The water used for diluting it must be neutral, or only slightly alkaline, and must be nearly, or quite, free from salts.

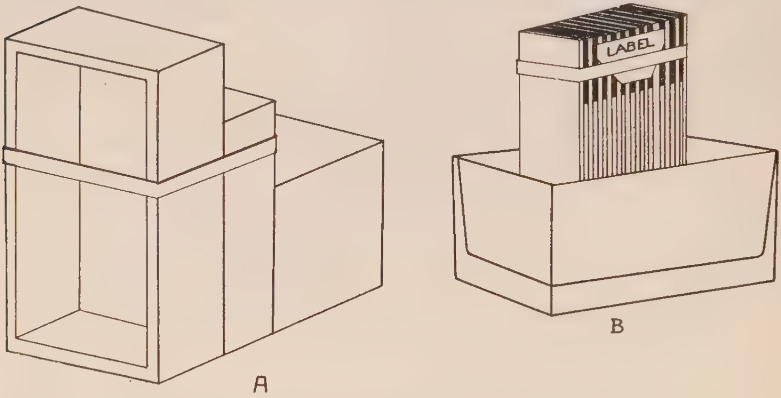

Fig. 20.—A, Slide-box held against supporting wooden blocks. B, Block of slides, with group label, in staining-dish.

The stock Giemsa solution may be made up by the following formula: Dissolve Azur II eosin, 0.3 gram, and Azur II, 0.08 gram, in twenty-five cubic centimeters of pure anhydrous glycerin at 60° C. Then add twenty-five cubic centimeters of absolute methyl alcohol (C.P., acetone-free) at the same temperature. Allow the glycerin-methyl alcohol solution to stand overnight and then filter.

The Azur II may be omitted from the formula of a Giemsa solution to be used for thick films. According to Giemsa (*Centralbl. f. Bakteriol.* I Abth. Orig., 1924, Vol. 91, No. 5, pp. 343-346) a glycerin suitable for this stain should have a specific gravity of 1.26 and a water content of only 1.5%.

In recent years we have been using a Giemsa solution ready prepared. We prefer Azur eosin, Gruebler. (This stain may be obtained in America of the American Kreuger & Toll Corp., New York, under the name of "Azur Eosine Solution for Romanowsky Giemsa Staining." An excellent Giemsa solution can also be obtained from Dr. Karl Hollborn, Leipzig.) Stock solutions, if kept in well-stoppered containers, remain in good condition for months, even in the tropics.

Dehemoglobinization previous to staining or fixation of films is unnecessary. Neither alcohol nor any other fixing agent should be allowed to touch the thick films before dehemoglobinization, and are not used at all in the method described below.

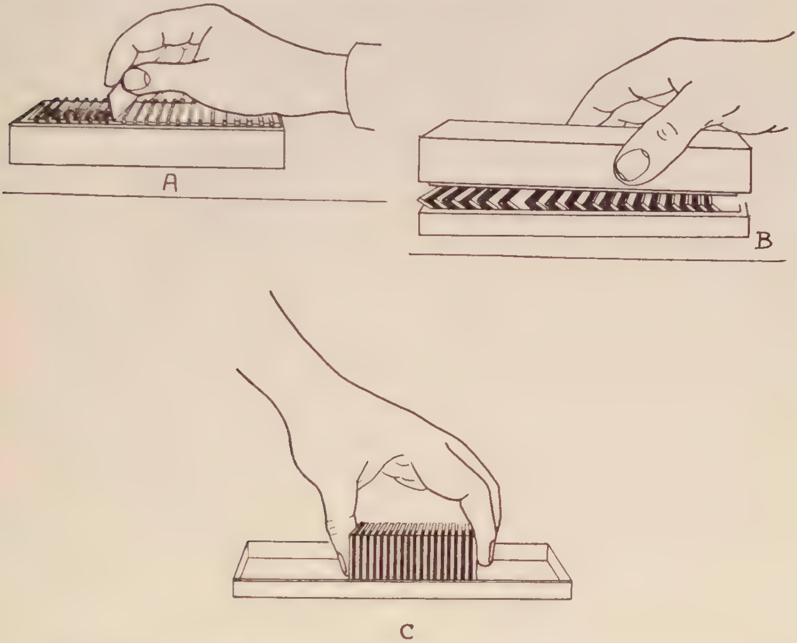

FIG. 21.—A, placing pasteboard separators, between slides in slide-box. B, Box inverted and raised, leaving slides and separators in lid. C, Assembling slides and separators into a block.

If only a few slides are to be stained, a Coplin jar or other convenient staining dish will serve. If a large number are to be handled, the methods illustrated by Figs. 20, B., and 21, A, B, and C, will be found very convenient.

Place all slides in the slide-box with the blood-films on the left and the wax-pencil numbers on the right. Pieces of pasteboard, each about 1″ × 1¼″ × 1/20″ thick (25 × 30 × 1.2 mm.), are dropped between the numbered ends of the slides, one to each interval. The lid is replaced, the box lifted and rotated clock-wise and inverted. The box is then raised from the lid, leaving the slides in the lid, separated from each other by the pieces of pasteboard. All the slides from a box, or any group of them, may now be easily assembled, fastened together by means of a stout rubber band, and given a group label.

To stain one block of twenty-five slides, place sixty or seventy drops (about 1.3 cc.) of Giemsa stock solution in a clean glass vessel and pour in seventy-five cubic centimeters of water. The pouring in of the water insures sufficient mixing. Then stand the block in the diluted stain and leave for about one hour. Any clean glass or porcelain dish will do, provided it is of such dimensions that the stain will cover the thick films to the top, and not wet the pasteboard slips.

Dilute the stain immediately before use and use it only once. To dilute the stock Giemsa solution, use distilled water, neutralized or at most but slightly alkaline (pH 7.0 to pH 7.2). Rain water caught in the open (not from a roof), melted snow, or ice made from distilled water, will serve, especially if boiled until free from carbonic acid. In any case, see that the water does not contain free acid. If it is not convenient to make a hydrogen-ion determination, a single indicator, phenol red, neutral red or an alcoholic solution of hematoxylin will serve. One may neutralize with a one per cent solution of sodium or potassium hydroxide or with dilute hydrochloric acid. Distilled water is often acid, and it is well to test the reaction of any water before using it. A tap water not too heavily impregnated with salts may give good results, but salts (especially sodium or magnesium chloride) tend to precipitate part of the stain. The sediment remaining in a dish after a previous staining may also precipitate part of the stain. A good practice is to rinse each staining dish immediately after use.

Occasionally, INFUSORIA become so numerous in a supply of water long kept in the laboratory that they appear in the thick films and may confuse the inexperienced by their resemblance to parasites.

Decolorization after staining is not always necessary. A sufficient decolorization is usually obtained by setting the block, while the slides are still wet from the stain, for five minutes in clean water, preferably the same as that used for diluting the stain.

These staining directions will serve for most cases, but a good deal of latitude is permissible if one observes the essentials—a good Giemsa stain and a proper diluting water. The length of time for staining as well as for decolorization may vary with the dilution of the stain, the amount of stain per slide and the average thickness of the blood films. A very little experience with known positives will serve as a guide. The red of the chromatin of the parasites and the blue of the cytoplasm should stand out distinctly against the background.

When the background is deep blue and the leucocytes almost black the preparation is overstained and the red of the chromatin may be somewhat obscured. Overstaining is apt to occur when only a few slides are left for a full hour in a large amount of stain. When the leucocytes in the thicker parts of the film are pale, the preparation is understained. Sometimes there is enough well-stained area at the margin or in the thinner parts of a preparation to serve, but, especially when a whole batch of slides is understained, it is best to restain.

Two or more blocks can be stained at once in a larger dish. In the staining and decolorizing specimens an interval timer clock will prove to be a useful monitor.

After staining the block may be placed on a piece of blotting paper to drain and dry at room temperature or in the incubator. It may be then set away in a dry, dark place free from dust and kept for weeks before examination. When the blocks are to be kept for some days it is best to replace the rubber bands with string, unless very strong bands have been used.

During the whole process of staining, and during storage, the position of the slides in a block is not changed, so that they remain in the same order in which they were taken.

Thin films may be made on the same slide as the thick, and the slides labelled with an ordinary lead pencil in the thin film. In ordinary surveys it is not necessary to make thin films of all cases. When they are employed they need not be stained unless they are desired for some particular purpose—confirmation of the results obtained in the thick-film, for example. Then they may be stained by the method of Wright, Leishman or any thin-film technique preferred. A thick line made with a wax pencil may be drawn across the slide to keep the stain used in the thin film from spreading over the thick. We have found Pappenheim's Panchrome (obtainable from Karl Hollborn, Leipzig), diluted with water and used as one does Giemsa, an excellent stain for thin films. As a routine, thin films should be fixed with methyl alcohol before staining. But thin films long dried will afford good specimens for diagnosis without fixation.

Quick staining of thick films for early diagnosis. Spread the blood, or a part of it, a little more thinly than for ordinary thick films. Dry. Lay the slide flat, film side up, and pour on a generous quantity (3 or 4 cc.) of freshly diluted Giemsa stain. Stain fifteen or twenty minutes. Wash with water cautiously so as not to loosen the film. Dry and examine. Films brought in one or two at a time for diagnosis are often best stained by this method, especially a kind intended for a thin film but having a surplus heap of blood at one end or the other of the thinner part.

Preservation of films after examination. If it is desired to preserve a preparation, warm the slide, wash off the immersion oil with xylol, and quickly wash off the xylol with absolute ethyl alcohol. Blot or dry quickly. Cover the films with liquid petrolatum or petrolatum (vaseline) and keep them away from the light. The alcohol is used to prevent the formation of a ground-glass deposit which sometimes occurs after the use of xylol alone; and if a cold slide is washed with xylol and alcohol enough water may condense on it to dilute the alcohol and partly decolorize the film. Where one wishes to avoid the use of cedar oil for immersion, liquid petrolatum, heavy (U.S.P. IX) will prove to be a fair substitute. In that case the same medium will serve for examination and preservative.

Advantages of the thick film method, especially for malaria surveys, have been recognized by all who have given it a fair trial. An assistant may be easily taught to collect good specimens, and the method has been widely and successfully used in field work. Much time is saved in the examination of specimens. When parasites are at all numerous they are usually picked up in the first thick-film field; when they are rare, they are often detected in the thick film when they might have been missed in a thin or found only after a long search. The chief purpose of the thick film is, of course, the diagnosis of malaria rather than the study of the characteristics of malaria parasites, a purpose for which the thin film is more suitable.

Recognition of parasites in the thick film. The difficulty of learning to recognize parasites in the thick films has been overestimated. Examiners familiar with the appearance of parasites in the thin films have learned to do thick films in a day or two. The following directions may be of use to the less experienced. It is assumed that the examiner is already familiar with the appearance of malaria parasites in the thin film.

Examine thick films of normal blood and get familiar with the appearance of all normal constituents of the blood as they appear in such preparations. Make thick films of known positives, spreading the blood at one side of the drop until it becomes essentially a thin film. Before staining, dry the preparation enough to partially fix the red blood cells at the thinner margin. Compare the appearance of parasites in portions of the film of various thickness. A little practice of this sort will teach the learner more than can be expressed in many paragraphs of description.

A few general directions may be of particular assistance: Except in the case of crescents, it is unsafe to call anything a parasite unless it shows a red chromatin dot, or mass, associated with blue cytoplasm. The latter, of course, is not always in the form of a ring; it may appear as a round or oval body and is not uncommonly irregular in form. With increasing experience and in clean preparations one can sometimes take into consideration pigment alone or pigment associated with cytoplasm only.

Bacteria sometimes stain like chromatin and may deceive when accidentally associated with a basophilic cloud or a remnant of a leucocyte; but bacteria are rare in clean preparations and do not show the even distribution throughout the film characteristic of malarial parasites. Red dots or masses, not bacteria, occasionally occur evenly distributed in the blood, and in some cases may be the remnants of malarial parasites; but it is not safe to reckon them as parasites unless they are clearly associated with cytoplasm. These red dots are found in the red cell itself, and in the thick film may lie close to the blue remnant of a basophilic cell. As in the case of bacteria, one has to be on his guard against regarding such accidental associations as parasites. A good general rule is not to reckon as a parasite anything which can be interpreted as an artifact. The number of doubtful appearances will diminish as one gains experience.

When dirt from the skin is mixed in the thick film, much time is lost in trying to distinguish parasites from bacteria and other débris, and errors in diagnosis are far more likely to occur. If a preparation is very "smudgy," especially if the dirt is distributed throughout the whole film, it is best to discard the specimen and get a new one.

Identification of the species of parasite in thick films. The red blood cells throughout most of the thick film are laked out, so that they no longer serve as a guide in the determination of species. On the other hand, the thick film often affords parasites in large numbers and in a greater variety of stages of growth than one would find in the ordinary examination of thin films. When parasites are abundant enough it is well to examine the margin of the thick film where the red cells are partially fixed by drying. In taking films it is a good plan to make a small, thinner extension to the thick drop or to place a thin dab of blood beside it. These thinner places become sufficiently fixed by drying and may be stained along with the thick part.

A few general directions may assist in the identification of species in thick films:

Benign tertian (*P. vivax*): In most specimens these parasites appear in different stages of growth and one can find older schizonts (plasmodia) easily recognized by their larger size, irregular form, abundant chromatin and pigment. In some thick films the outline of the enlarged host cell persists, even at the center of the preparation; at the margin of the preparation the host cell often remains intact and may exhibit Schüffner's dots. Benign tertian rings or younger parasites usually have larger chromatin dots and more abundant cytoplasm than do estivo-autumnal rings. When the cytoplasm occurs in ring form, its outline is less regular than in estivo-autumnal.

Quartan (*P. malariæ*) is regarded by some authors as hardly distinguishable from benign tertian in thick films. In most cases, however, the smaller and more compact rings and schizonts, and the most abundant pigment, serve to distinguish them. In sporulating forms the quartan has only eight spores while the benign tertian has sixteen or more.

Estivo-autumnal (*P. falciparum*): Rings may vary greatly in size, but are generally smaller than those of benign tertian. Chromatin dots are smaller and more often two dots occur in one ring. Where many small rings and no plasmodia are seen, it is usually safe to identify the parasite as estivo-autumnal. Crescents, characteristic of estivo-autumnal, are easily recognized when typical in form. But they sometimes change their shape in slowly drying films and may assume more rounded forms, which may simulate the schizonts of benign tertian or quartan. The more compact pigment of crescents, the deeper staining of the cytoplasm, and, in some cases, a pink remnant of the host cell will aid in identifying doubtful forms. At the margin of thick films crescents dry more rapidly and are more apt to retain their typical form. Except crescents, larger parasites of estivo-autumnal are very rare in the peripheral blood.

Mixed infections are commonly overlooked in any kind of film unless the parasites of the two or more species occur in some very characteristic form. Thin films may show the specific characters more clearly, but, more often than thick films probably, fail to disclose a mixed infection, since fewer parasites are commonly examined. In any case it is well to continue the search after the detection of the first parasite. In any sort of preparation where only a few young rings are present, it is sometimes impossible to identify the species.

We generally use the thin film for identifying and estimating the number of gametocytes of benign tertian and quartan. In the case of crescents, the thick film is more suitable.

Minimum equipment for examining thick films. Any standard microscope fitted with a good mechanical stage is suitable. Expensive apochromatic objectives are not necessary, but much greater flatness of field and increased definition may be obtained by the use of a good fluorite objective, such as the Bausch and Lomb No. 1043. It is a good plan to use an eyepiece of relatively low magnification, such as a 5X, as a searcher, but also to have a good 7.5X eyepiece to give higher magnification for examination of doubtful parasite appearances. The usual small substage lamp is inadequate for illumination; the chalet type of lamp fitted with 100-watt electric bulb and daylight glass window is to be preferred.

Length of time to devote to thick films apparently negative. It is commonly recommended that fifteen to twenty minutes be devoted to a thin film before it is declared negative, and five minutes to the thick film. In either case the time spent on apparently negative specimens must vary with circumstances. When, for example, the sole purpose is to find a crescent carrier suitable for mosquito-infection experiments, a fraction of a minute will suffice for the thick film. In a clinical case it may be necessary to spend a good deal of time on a film, but here it is usually possible to get a new specimen taken at a time when parasites may appear in larger numbers.

The question of time of examinations is usually most important in connection with malaria-parasite surveys. It is difficult to establish a standard time since the skill of individual examiners varies so greatly. We recommend that each examiner determine for himself the amount of error incident on shortening the time devoted to thick films apparently negative. Select a batch of slides where a fair percentage, say twenty per cent or more, of positives may be expected. Note the amount of time required to find the first parasite in each positive, and continue the examination of negatives for ten minutes. It then becomes possible to estimate the error which would have been incurred had the time been shortened. The skilled examiner will probably find that a very small percentage will be missed if the examining time be limited to three or even two minutes. The experiment may be worked out in terms of the number of fields examined instead of the time devoted to the search. When basophilia or other evidence of anemia, or suspected remnants of parasites are found in a preparation, the examination is prolonged.

If a survey of a given group of persons be repeated after a few days one usually obtains new positives among former negatives; the best single examination can disclose only a fraction of the persons actually infected. But a single survey gives results of much comparative value, especially if the examiner follows the golden rule of recording as negative all doubtful specimens. The examiner is then sure that the population contains at least the percentage positive which he has obtained, and if he has ascertained his examination-time-error and has adopted a standard time, he will obtain results reliable for comparing the malaria rate of one population with that of another. It is of questionable utility to attempt to prescribe an examination-time applicable to all examiners and all circumstances.

Much time can be saved in collecting blood specimens if the slides, boxes, and other apparatus are placed before the collector in convenient order. The collector sits alongside a table, near the end. His right side should be nearest the table. The slide box is set upright a little to the left and in front of the collector. The cover of the slide box is placed next in order to the right, and the numbered slides are ranged in consecutive order, numbered side down, upon the box cover. The pricking-needle, well fixed in a large cork, is placed within convenient reach in front of the slide-box. The subject stands to the left of the collector, and the blood is taken from the middle finger of the left hand. The blood films are placed in the box film side down, the numbered ends of the slides all toward the left, and films toward the right.

In collecting blood specimens, one can save a great deal of time by the use of cards instead of a note-book for recording data. In surveys of schools cards may be distributed among older children and each can be directed to write his name, age, residence or other information. Sometimes these data are more accurately recorded by the child than by the teacher. The child brings his card to the examiner, who takes the blood specimen and enters the slide number on the card. After the results of the microscopic examination has been recorded on the cards, these may be classified by age of child, type of parasite or any other datum. When these results have been tabulated the cards may be arranged alphabetically.

Any attempt to prevent expense by the use of inferior stains, slides or other necessary apparatus is likely to prove to be poor economy. The time lost in collecting and staining an unserviceable batch of specimens may be worth more than a month's supply of a proper stain.

CHAPTER XXXVI

LABORATORY METHODS IN MALARIA: THE INFECTION AND DISSECTION OF MOSQUITOES

By

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Anopheles are infected with malaria in the laboratory for various purposes: to determine the susceptibility of an anopheline species to malaria, to measure the effect on gametocytes of a drug or treatment, to obtain infected mosquitoes for the treatment of progressive paralysis, or to obtain material for teaching purposes. The requirements of experiments will vary more or less according to the purpose for which they are made; but in nearly all cases it is necessary to have a plentiful supply of mosquitoes available for feeding on gametocyte-carriers.

Anopheles may be collected in the adult stage in daytime resting places, where they often occur in large numbers. Such mosquitoes are suitable for some experiments; but in a malarious region specimens may be already infected and may thus introduce a serious error into the experiments.

Mosquitoes which have emerged in the laboratory are more suitable for infection experiments. They are best collected in their natural habitat when they have reached the stage of full-grown larvæ or pupæ. Small larvæ may be reared to normal maturity in artificial breeding places, but it is usually possible to find a natural breeding place where older specimens are already present.

A great variety of mosquito cages have been described. I prefer for most purposes a cage made of a small glass lantern-chimney, about four inches in diameter and four inches high (Dietz "Little Wizard," clear glass). The ends are covered with bobinet or other mosquito netting. If the chimney does not have a rim for tying on the bobinet, one can be made by laying a thick piece of twine lengthwise on a long strip of adhesive tape. The tape, covering the twine, is then placed around the cage. A hole is made in the bobinet at one end for putting in or removing mosquitoes. This opening may be plugged with cotton.

Mosquitoes emerging from a collection of pupæ are conveniently got into a cage in the following manner: Place the pupæ with water in a tea cup or similar receptacle and upon it set a lantern-chimney cage from the lower end of which the bobinet has been removed. The rim of the cage must fit the cup closely. As the mosquitoes emerge they will fly into the cage. When a sufficient number have entered, remove the cage and replace the bobinet.

Mosquitoes will soon die unless they are fed. If a suitable gametocyte carrier is available they may be fed on him the day after their emergence. Some species bite well on the day they emerge, but usually feed much better a day later. But suitable gametocyte carriers are not found every day and may not long continue to carry gametocytes in sufficient numbers for infection. Therefore, unless one has a prolific and convenient breeding place of *Anopheles* available, it may become necessary to keep adult mosquitoes in stock while awaiting a suitable carrier. A batch may be kept alive and in good condition for long periods by feeding them every four or five days on a normal human subject or a laboratory animal. It is advisable to have several batches on hand, feeding them on different days so as to have a hungry batch ready in case a carrier is found. In summer weather mosquitoes bite well about two days after a blood meal and may then be used on a carrier.

Method of feeding mosquitoes on a carrier. It is a good plan to moisten the skin of the patient before the cage is applied. The cage may be placed on the arm or any part of the body of the carrier. He may be persuaded to hold the cage in his hands with the palms in contact with the bobinet at both ends. Most *Anopheles* bite well, especially if strong specimens are used and are allowed to get thoroughly hungry before feeding. They usually bite better if deprived of their water supply some hours before feeding and allowed to get thirsty. Sometimes one can stir up the less eager specimens by breathing into the cage. *Anopheles* can be fed at any time during the day.

The conditions of some experiments require that mosquitoes be fed but once and only those which have taken blood be dissected.

In such experiments the unfed mosquitoes must be separated from the engorged ones. For removing living mosquitoes from a cage, a device (Fig. 22) will be found very serviceable, and can be easily made in the following way: Cover the end of a thick-walled rubber tube with a piece of bobinet and push it two or three centimeters into a glass tube, in which it should fit closely. The rubber tube should have a lumen six or eight millimeters in diameter; the glass tube may be made by cutting off the closed end of a long narrow test-tube. The rubber tube is held firmly in place by a strip of adhesive tape wrapped around the junction of the two tubes. In using the device, the end of the rubber tube is placed in the mouth of the operator and one or several mosquitoes sucked into the glass tube, which may then be closed with a cotton plug.

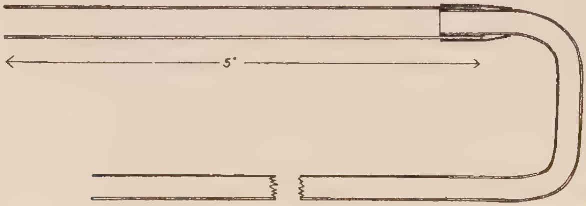

Fig. 22.—Device for removing living mosquitoes from a cage. Shown in longitudinal section.

The insects are conveniently examined in the glass tube, under a magnifier if desired, and subsequently blown into a new receptacle.

One must be careful not to handle freshly engorged mosquitoes too roughly, so it is usually best to leave them in the cage and withdraw those which have not fed. It is easy to distinguish fully engorged mosquitoes while in the glass cages.

After the mosquitoes have fed on a carrier the cages are placed on a shelf and tilted slightly on a piece of wood or some small object to allow a free circulation of air in the cage. To insure constant moisture, a piece of damp cotton is kept on top of the cage. In a very dry climate it may be advisable to place plates of water or moist cloths in the cabinet where the cages are kept. A cupboard or meat-safe with screened doors makes a suitable receptacle for storing cages. It should be made ant-proof, else the entire collection of mosquitoes may disappear overnight.

Mosquitoes will live for five or six days after a blood meal without any new food. If the infected mosquitoes must be kept for a longer period it is advisable to give them a new feeding on a guinea-pig or other laboratory animal. Some workers place a piece of raisin or other fruit on the cage to supply food, but such material seems to promote the growth of yeasts or bacteria in the stomachs of the mosquitoes. If any food other than blood is used, white sugar seems to be the most suitable. It may be conveniently supplied to the mosquitoes by placing a small amount on a piece of moist filter paper which is then laid on top of the cage. Mosquitoes are usually kept at room temperature while the malarial parasites are growing.

Uninfected mosquitoes kept in stock while awaiting use are cared for in the same way as the infected batches. They may be kept at lower temperatures in which they live longer and require less food.

Cages should be kept free from dead mosquitoes. Sometimes many of the mosquitoes will die, whatever precautions are taken; but it is usually possible to preserve a large proportion of them, even when kept at room temperature in the tropics. Mortality is apt to be higher during the first few days. Those which die after the second day may be removed and dissected, since oöcysts are then usually large enough to be discernible, especially in batches of mosquitoes which have been kept at temperatures of 70° to 85° F. Dead mosquitoes if not left at room temperature more than half a day usually remain in good condition for dissection and examination. In no case should they be allowed to dry, as dissection then becomes very difficult or impossible. A good routine is to remove the dead mosquitoes morning and evening for dissection. Those removed in the evening may be kept on ice and dissected on the following day.

If no positives appear among the dead mosquitoes, it is advisable to begin dissecting the living ones on the fourth or fifth day after feeding on the carrier, a time when oöcysts are large enough to be very easily seen. If the whole batch should prove to be negative, one is then spared the trouble of keeping them longer. Should a positive appear, one may keep the remainder alive until the later stages of parasites are developed. Dead mosquitoes may be easily removed through the opening in the bottom of the cage by means of small forceps. The living ones are removed by means of the device described (Fig. *22*), placed in a series of cotton-plugged tubes, one or two per tube, and chloroformed as needed.

A light treatment with chloroform is preferable. The chloroformed ones are easily spilled from a test tube on to the slide for dissection. Living mosquitoes which are not to be dissected may be returned to the cage. Mosquitoes ready for dissection are conveniently kept in a Petri dish having a moist piece of filter paper on the bottom.

Dissection of stomachs for oöcysts. Use well cleaned slides free from grease. Place a drop of normal salt solution midway on the slide but near the edge away from the observer, and into it place the dead or anesthetised mosquito. Under the lens, remove the abdomen by means of the dissecting needles. The abdomen may be cut off cleanly; but it is a better plan to dissect it away so that it is opened widely at the anterior end, especially in specimens full of nearly mature eggs. Break the integument of the abdomen near the posterior end, leaving one or two of the terminal segments attached to the gut. Place the point of one dissecting needle on the integument of the abdomen and the point of the other on the terminal segments. Separate the stomach from the wall of the abdomen, using a very slow traction. One may either pull away the abdomen or pull out the stomach from it.

With a needle placed on the terminal segments, pull the stomach to the middle of the slide. It is easily drawn over a wet surface. With a small piece of filter paper wipe away the drop of salt solution in which the abdomen was dissected together with the eggs and other débris, leaving a thin film of salt solution around the stomach. Brush back the Malpighian tubules with the needles so that they will not overlie the stomach. They remain in position if the liquid is shallow. Place a small coverglass over the stomach with one edge resting on the upper part of the terminal segments. If there is not enough salt solution to surround the gut, add a little at the margin of the cover.

If the cover rests on the terminal segments the stomach will not be too much flattened. Oöcysts will appear on a rounded surface, and the stomach may be easily rolled into a new position by moving the coverglass laterally with a needle. If more flattening of the stomach is desired, the cover may be pressed down. If the terminal segments are not present, it is best to mount the stomach in a larger amount of liquid. One can then flatten the stomach by drawing away part of the liquid by means of a piece of filter paper placed at the margin of the coverglass.

In case the head has been removed for dissection of the salivary glands, it is possible to draw out the midgut without removing the abdomen from the thorax; but it is usually best to remove the abdomen in order to draw out the stomach more easily. If the hindgut breaks during traction, it is almost always possible to pull out the stomach from the anterior end of the abdomen without injuring it.

When the probabilities of finding an infected stomach are small, as is usually the case when mosquitoes caught in the wild state are examined to determine natural infection, much time can be saved in both dissection and examination by dissecting three or more specimens in the same drop of salt solution, and covering the stomachs with one coverglass. If a positive specimen appears, it may be separated from the others.

Very serviceable dissecting needles may be made by inserting large sewing needles (No. 10) in a stick or in a special holder. The points may be easily ground into the form of small blades. Small thin covers are preferable for mounting stomachs. I prefer for the examination of single stomachs a square cover, about five millimeters square and No. 1 in thickness. It is possible to use fragments of large covers, but it is far better to order special sizes from the dealers.

A binocular or other dissecting microscope may be employed. Since only a small magnification (six or seven diameters) is needed for dissection, a simple lens mounted in a lens-holder will serve. I have found a simple lens, held in a lens-holder provided with ratchet and pinion and a long movable arm, a very convenient apparatus, since it is portable and allows abundant working room. A thick board or an ordinary wooden slide box will serve as a stage, a portion of which is blackened to afford a dark background for dissection. A small fine whetstone, fastened to the side of the stage with its upper surface flush with the surface of the stage, has proved very convenient for cleaning or sharpening needles under the lens.

Identification of oöcysts. Oöcysts are more or less spherical in form and contain denser protoplasm than do gut cells. They are attached to the stomach wall and can often be distinguished by their position from stomach cells, yeast cells or other bodies which lie within the stomach. A characteristic very useful for identification of oöcysts is the pigment, which is easily found in oöcysts except in those in more advanced stages. The pigment varies somewhat with the species of malaria parasite, but always appears as a clump or chain of granules varying in color from brown to almost black. They appear in sharp contrast when the diaphragm of the substage condenser of the microscope is opened widely.

Sometimes fat droplets or immature ova underlie or overlie a flattened stomach and may simulate oöcysts. These will not be attached to the gut and may often be removed by alternately lifting and lowering the coverglass, a new drop of salt solution having been added at its margin. Or the coverglass may be moved laterally by means of a needle, preferably under microscopic control, thus rolling the stomach until the doubtful body appears at the side. It is usually possible to identify oöcysts on the flattened surface of the stomach without rolling it.

Dissection of salivary glands. The salivary glands are found in the upper part of the thorax a little below the neck. A method in common use for dissecting out the glands is as follows: Remove the legs and wings of the mosquito and place it in a drop of salt solution. Hold the thorax with one needle, place the other between the head and thorax and with it draw the head slowly from the body. The glands often come out attached to the head and may be cut away from it with the needle. If they do not come out, they may be pressed or dissected out from the portion of the thorax in which they lie.

I have devised a technique which offers some advantages. Remove the legs of the mosquito and cut off the head cleanly from the body. The wings need not be removed, especially if they project backward. These operations can be quickly done on the slide by means of needles with points ground to sharp blades. Place the specimen on its side in a very little salt solution with the thorax to the right of the observer. By means of a pair of fine forceps held in the right hand pick up a small square cover, about five millimeters square, and slope it on the thorax (Fig. 23). Keep the mosquito in position with a needle held in the left hand. Press down the cover with the forceps in such a way as to squeeze out the contents of the thorax situated just below the neck. As the coverglass descends withdraw the thorax by means of the needle held in the left hand.

The glands almost always come out and are found well under the coverglass. They usually appear in a single group. If they are mixed with more opaque material from the thorax, they may be washed free by lifting the left side of the cover at which a little salt solution alone is sufficient to hold the coverglass in position intact, the coverglass should not be pressed down closely; but

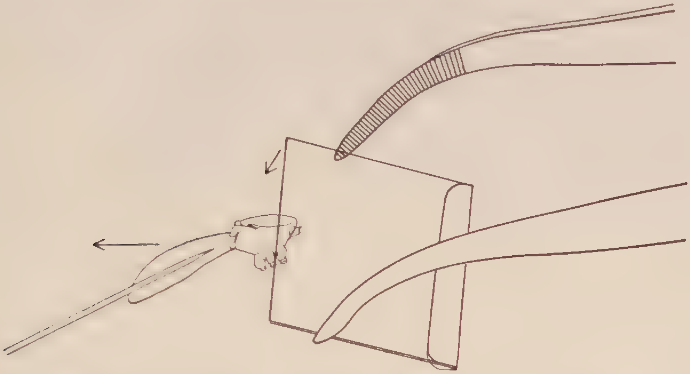

FIG. 23.—Position of mosquito, coverglass, forceps and needle in the process of pressing out the salivary glands.

sporozoites may be easily recognized when partially pressed out from the glands.

It is more convenient to remove the salivary glands while the abdomen is attached, but it is quite possible to press them out after the removal of the abdomen for the dissection of the stomach.

The whole process is made a little easier if the coverglass is first set up at an angle on the slide and held in position by a very small amount of stiff balsam. Then the coverglass may be pressed down with a needle after the thorax is in place, the balsam acting as a hinge. The use of balsam, however, is hardly necessary. The salt solution alone is sufficient to hold the coverglass in position when it is sloped on the thorax; one may then release the grip of the forceps on the margins of the cover and press it down with a tip of the forceps or with a needle.

Sporozoites are usually recognizable by their form and their position in the salivary glands, but it is always advisable to confirm the diagnosis by the use of some Romanowsky stain, which colors the chromatin of the sporozoites red and the cytoplasm blue or violet. In preparing the preparation for staining, the coverglass may be lifted and placed, wet side up, over a very small drop of balsam, which is placed on a dry part of the slide. The balsam holds the coverglass firmly in position. The whole preparation is then dried, fixed with methyl alcohol, and stained with Giemsa as one would a thick blood-film. Or, one may stain by Wright's or any thin-film method. All or the most of the sporozoites remain on the slide or the cover.

When mosquitoes caught in the wild state are examined to determine the percentage infected with sporozoites, a saving of time in dissection is very desirable, since the percentage infected is usually very small, and large numbers have to be examined in order to obtain significant figures. Some workers (Sergent, E. and E., 1912) dissect out on a slide the contents of that portion of the thorax in which the salivary glands lie, dry the material, fix with methyl alcohol and stain with Giemsa. Smears from many mosquitoes can be made on a single slide and may be stained and examined at leisure.

I have had practically no experience with this method. It seems to be a good one for detecting at least the minimum percentage of infections, for it is very probable that sporozoites, if present in large numbers, would be found in preparations made in this way. It would appear desirable that the salivary glands, or at least portions of them, should appear in negatives as evidence that the dissections were properly made.

Permanent preparations of stomachs or salivary glands. The preparation of material for cytological or other detailed study of mosquito material demands special methods which cannot always be attempted in ordinary mosquito infection experiments. But it is often desirable to preserve material sufficiently well for class or other demonstration purposes, and a few directions for making permanent preparations are given. It is presumed that the reader is familiar with the ordinary technique of preparing tissues, in sections or bulk, for microscopic examination, or has access to one of the numerous manuals which describe such methods.

Unstained specimens of stomachs and of salivary glands may be mounted in dilute formalin under sealed coverglasses. A special preserving fluid is recommended by MacGregor:

5% solution of borax	10.00 cc.
40% solution of formaldehyde	10.00 cc.
Glycerin	0.25 cc.
Water to make up	100.00 cc.

Specimens may be mounted fresh or first fixed by formalin or other fixative. Schaudinn's fixative is apt to make the unstained specimens too opaque.

It is usually unnecessary to prepare a cell beforehand for enclosing such small objects as stomachs or salivary glands of mosquitoes. Sometimes it is advisable to place a bit of thin coverglass or some small object with the specimen to prevent too much flattening by the coverglass. If the specimen is first attached to the slide by some fixative it becomes easier to mount it. The specimens should be mounted free from air bubbles. The excess of fluid should be removed from the preparation, and the slide at the edge of the coverglass well dried before applying the cement. I usually make the first ring of asphalt cement or of a cement of similar consistency. When this has dried I superimpose a second ring of soft paraffin. This is conveniently made by melting together equal parts of paraffin and liquid petrolatum (Liquid Petrolatum Heavy U.S.P. IX) and should be liquefied by heat and applied when quite warm. A ring of some firmer cement may then be added as a protection to the paraffin ring.

I have devised a method of making permanent preparations of unstained material, easily carried out and very serviceable for the examination of specimens under low magnification. Take up the stomach or salivary glands in a small pipette and place them in a minimum of fluid at the side of a clear glass vial about forty millimeters long and five millimeters in diameter. The thinner the wall of the vial the better. It should be well cleaned before use. The excess fluid may be drawn away by means of a pipette or a narrow strip of filter paper inserted in the vial; and the specimen may be arranged under the lens by means of a bent dissecting needle. Place in the mouth of the vial a cotton plug moistened with a forty per cent solution of formalin or other vapor fixative. Place the vial mouth downward. The vapor will fix the specimen to the side of the vial as the fluid drains into the cotton plug. The vial may then be filled with dilute formalin or any preserving fluid desired, and tightly stoppered with a rubber or other impermeable stopper, taking care to avoid the inclusion of air bubbles.

To examine the specimen microscopically, fasten the vial temporarily to a slide with a strip of adhesive tape and examine under any objective with sufficient working distance to focus through the wall of the vial. The image appears much more distinctly if a drop of liquid is placed on the vial over the specimen and covered with a coverglass. The cover may be temporarily held by cedar oil or permanently cemented on with balsam. Better illumination is obtained by placing balsam or other liquid between the vial and the slide. The vial may be permanently sealed to the slide or kept separate. A flat vial can be employed or one can make vials of various forms out of glass tubing.

This method of preservation is serviceable for the intestinal tracts of mosquito larvæ or for any small object that can be made to stick to glass. Large oöcysts on infected mosquito stomachs or any detail of the specimen not requiring very high magnification can be demonstrated in these preparations. Several specimens may be mounted in the same vial. The preservative can be changed at will and if air appears in the vial it can be easily removed. If the specimens come loose from the side of the vial they are not lost.

The method of mounting in vials is designed primarily for unstained material, but one may use Schaudinn's or other liquid fixative and subsequently wash, stain, dehydrate and clear the specimen in the vial, finally filling the vial with liquid petrolatum. Specimens may be stuck to the side of the vial by Schaudinn's fixative (see method described below for fastening mosquito stomachs to slides).

In case it is necessary to examine specimens under the higher powers of the microscope, either sectioned or stained *in toto,* it is advisable, of course, to fix them and eventually stain and mount them on slides in balsam.

Stomachs or salivary glands are dissected out before fixation. A preliminary fixation on the slide makes them much easier to handle. They may then be transferred by means of a needle or a small camel's-hair brush to a watch-glass or vial where they receive further fixation. They are then washed free from fixative and run up through increasing strengths of ethyl alcohol to seventy per cent, where they may remain indefinitely. In case Schaudinn's fixative is used, the stomachs or salivary glands may be transferred directly from the fixative to seventy per cent alcohol and washed in several changes of this alcohol until free from the fixative. Add enough iodine (Weigert's or Lugol's solution) to one of the alcohols to facilitate the removal of the mercuric chloride. If the specimens are deeply stained in the iodine they may be decolorized by seventy per cent alcohol to which a drop of one per cent solution of sodium thiosulphate has been added. They are finally brought into pure seventy per cent ethyl alcohol. It is well to allow abundant time for washing, two hours at least.

Material may be preserved in the seventy per cent alcohol or stained at once. All the processes of staining, dehydrating and clearing may be carried out in vials or watch glasses.

Salivary glands or stomachs may be stuck to the slide by the fixative and subsequently treated as one would a section. Schaudinn's fixative will usually fasten specimens, at least the well flattened ones, to the slide if the following precautions are taken: Draw away the salt solution with a piece of filter paper until the specimen is almost dry. Add the fixative very gradually at first, so that fixation takes place while the object is in close contact with the slide. A drop of fixative may be put on the slide near the specimen and gradually drawn to it with a needle. In the case of stomachs, remove the terminal segments of the abdomen or dissect them out so that they will adhere well. The more material in contact with the slide, of course, the more likely the specimen is to adhere. After a few minutes of fixation transfer the slide to fresh Schaudinn fixative in a Coplin jar. Specimens are eventually washed, stained, dehydrated and cleared in Coplin jars or similar containers.

In case one wishes to demonstrate the position of oöcysts on the rounded wall of the stomach or to obtain material for sectioning, it is best to avoid too much flattening. A stomach may be examined for oöcysts without the coverglass. However, a stomach taken from a freshly killed insect will usually resume the rounded form after the coverglass is removed, provided it has not been flattened too much. Add new salt solution after the removal of the coverglass. A flattened stomach may be made to resume its rounded shape if the slide be gently heated while the stomach is submerged in liquid (MacGregor).

Stomachs or salivary glands may be fixed, washed, stained, dehydrated, cleared and mounted in balsam without removing the coverglass. Use a small, thin coverglass not over six millimeters in shortest dimension. A narrow rectangular cover is preferred. Draw away the surplus salt solution. Run the Schaudinn or other fixative under the cover, and "irrigate" by withdrawing the fixative from one margin of the cover with filter paper while supplying new fixative at the other. The coverglass should be kept under gentle pressure during fixation. A very simple device for doing this is made by cutting a triangle (Fig. 24) out of heavy pasteboard and inserting a bent pin at the tip of the acute angle. Rest the point of the pin on the coverglass while applying the fixative. The whole process is best done under the lens. After a few minutes' fixation under pressure, the slide may be set upright in a fresh lot of fixative.

When a considerable area of the specimen is in contact with both slide and cover, the cover usually adheres during the whole process of preparing these under-cover mounts. The thinner and smaller the cover the better the chances of its adhering. If the cover comes loose the specimen usually adheres to the slide. Slides should be gently lowered into a Coplin jar in order to avoid jarring the covers loose from them.

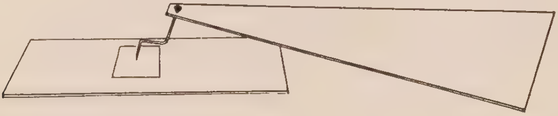

FIG. 24.—Pasteboard triangle for holding down a coverglass during fixation.

All of the processes of fixing, washing, staining, dehydrating and clearing under the cover must be greatly prolonged. The time allowed for each medium must vary with conditions, the most important of which are the size of the cover and the distance between the cover and the slide. One can safely pass from one medium to another of much higher or lower concentration, since liquids diffuse very slowly under the coverglass. There is little danger of overstaining, so that one may leave the preparation over-night in undiluted stains. When the specimen is in xylene, a small drop of balsam is placed at the margin of the coverglass. As the xylene evaporates the balsam replaces it.

Very good preparations can be obtained by this method. The specimen remains in the position in which it is first examined and it may be examined under the high dry objective at any time during the preparation.

Fixatives. In general, the best fixative for mosquito stomachs or salivary glands is that of Schaudinn, to which the acetic acid component is added shortly before use. Bouin's is also a suitable fixative. An hour's fixation will suffice for these small objects, but a longer fixation will do no harm. When the preparations are fastened to slides it is usually more convenient to wash them in water before transferring them to the alcohols for subsequent washing and dehydration.

Staining. Mayer's acid hemalum is an excellent stain for stomachs and salivary glands. It is precise and usually does not overstain. It may be used undiluted or diluted in distilled water. Borax carmine is also recommended for this sort of material.

In mosquito, as in other material, it is difficult to lay down definite rules for the length of time a specimen should be stained. The character of the material itself, its fixation and the dilution of the stain are important factors. It is always best to control the process by examination under the microscope while the staining is in progress.

A few general rules may be of assistance: "Free" specimens stain more rapidly than those fixed to a slide. Specimens stained under coverglasses should be kept at least over-night in acid hemalum. A light stain is preferable for the demonstration of oöcysts on a stomach wall since a heavy stain tends to obscure them. Stomachs fastened to the slide but without a coverglass may be sufficiently stained by a treatment of one hour in Mayer's acid hemalum diluted one to ten in distilled water. It is always necessary to treat specimens stained in hemalum with some alkaline fluid to fix the stain. Most tap waters, or alcohol slightly alkalinized by a few drops of a one per cent solution of sodium carbonate will serve. Counterstaining is usually unnecessary. If it is desired, preparations may be counterstained in one of the higher alcohols to which eosin or some alcohol-soluble dye has been added.

Dehydration and clearing of mosquito stomachs require a little more time than do sections. Material may be left a long time in the higher grades of alcohol. It is best to avoid passing from one medium to another of a much higher or lower concentration, especially in dealing with large hollow organs liable to collapse. In general, however, a specimen is not harmed by more abrupt transfers, provided it is well fixed and hardened. It is assumed here that the demonstration of finer cytological details is not required.

Labels may be written on the slide with a diamond pencil, preferably at the time of fixation. If desired, paper labels may be substituted after the preparations are stained and mounted in balsam.

Useful reference books for laboratory work on mosquitoes are: Stephens and Christopher's: *The Practical Study of Malaria and other Blood Parasites.* University Press of Liverpool. 1908. Wenyon: *Protozoology.* New York, William Wood & Company, 1927. MacGregor: *Mosquito Surveys.* London, Ballière, Tindall, & Cox, 1927.

CHAPTER XXXVII

EXPERIMENTS ON BIRD MALARIA

By

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INTRODUCTION

Avian malarial parasites are very widespread, and occur in many species of birds. For this reason and because they are easily transferable to canaries by either blood or mosquito inoculation, they offer an attractive field for research. The added fact that there are at least four distinct species offers an opportunity for comparison, and since both the parasites and the diseases they cause bear a close resemblance to the malarial parasites and periodic fevers of man the study of the avian malarias becomes genuinely important, the more especially because no one of the human malarias has as yet been successfully transmitted to any of the lower animals. It is hardly necessary in proof of this statement to point to the results of past work in this field, for the epoch-making discoveries of Ross and MacCallum will instantly come to mind, and the recent development of plasmochin therapy was directly due to long-continued bird experimentation.

THE PARASITES

1. *Nomenclature and History.* That malarial parasites may be found in the blood of birds has been known since 1885, when Danielewski first observed them, and the early investigators believed them identical with those of man. Although Celli and San-Felice described three separate species of malarial parasites as existing in birds, their description was not sufficiently careful to make these species recognizable to-day, and following a zoölogical study of the parasites published by Labbé in 1894, in which he

proposed the name *Proteosoma grassii* for the single species he recognized, the fact that there was but a single species of avian *Plasmodium* has been generally accepted. It has been evident for some time, however, that there are at least several distinct varieties of malarial parasites in birds, and in 1927 Hartman published a paper proposing that three distinct species be recognized on the basis of morphological differences. For these species, two of which he regarded as new, he proposed the following names: *Plasmodium præcox, Plasmodium cathemerium,* and *Plasmodium inconstans. Plasmodium præcox* he believed to be identical with an avian parasite described by Grassi and Feletti in 1890 under the name of *Hæmamœba præcox,* and therefore retained the same specific designation, although these two authors evidently regarded their parasite as the same as *Plasmodium falciparum* of man. These three species differ from one another chiefly in respect to their gametocytes (see Fig. 25), but there are also clear-cut differences in the type of infection which they produce in canaries, and in the length of their asexual cycles.

2. *Asexual cycles.* In the case of *Plasmodium cathemerium* the length of the asexual cycle is twenty-four hours, and sporulation occurs in the early evening, reaching its maximum about 6:00 P.M. The length of the asexual cycle in *Plasmodium inconstans* has not as yet been accurately determined, but is longer than that of *cathemerium,* and apparently less definite. There is also some doubt as to length of the cycle in *Plasmodium præcox,* although Hartman (1927) states that he believes it to be the same as in *cathemerium.* Taliaferro (1925) who first determined the length of the cycle in the latter species also worked with the so-called "Whitmore strain" (see below) and found the length of the asexual cycle of this strain to be thirty hours.

3. *Morphology.* The morphological differences are shown in the accompanying figures. It will be noticed that the gametocyte of *Plasmodium præcox* is an elongate, rather crescent-shaped affair, and that it does not displace the nucleus of the host cell. The gametocytes of *Plasmodium cathemerium* and *Plasmodium inconstans* are both of a round or somewhat irregular shape, and present no conspicuous specific characters, but the pigment granules of *cathemerium* are much coarser and somewhat rod-like, whereas those of *inconstans* are often nearly spherical, and very fine. In both cases the nucleus of the host cell is displaced and not infre-

quently entirely pushed out. The asexual forms of all three species resemble one another so closely that they are not easily distinguishable.

4. *Prepatent period.* There are also differences in the length of the prepatent period, and as above stated, in the infections produced. Huff (1927) found the prepatent periods to be as follows: *Plasmodium cathemerium*—5.78 ± 0.14 days, *Plasmodium præcox*—11.44 ± 0.45 days, *Plasmodium inconstans*—8.5 ± 0.37 days.

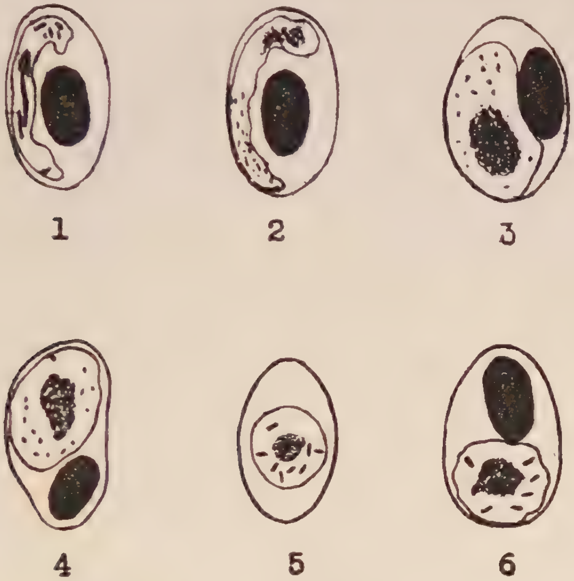

FIG. 25.—Gametocytes of the three species of avian malaria. 1. Microgametocyte of *Plasmodium præcox*. Note that the nucleus of the host cell is not displaced. Pigment granules appear at the ends of the parasite, and a rather ribbon-shaped nucleus in the center. 2. Macrogametocyte of *Plasmodium præcox*. The nucleus is shown as an irregular, somewhat granular mass at the upper end of the parasite. The cytoplasm of the macrogametocytes of all species takes a much heavier blue stain than that of the male forms. 3. Microgametocyte of *Plasmodium inconstans*. Note the small, almost dust-like pigment granules. 4. Macrogametocyte of *Plasmodium inconstans*. 5. Macrogametocyte of *Plasmodium cathemerium*. The pigment granules of this species are much coarser and more rod-like than those of the two species figured above. 6. Microgametocyte of *Plasmodium cathemerium*. Like the sexual forms of the other two species, the gametocytes of this one differ from one another chiefly with respect to the intensity with which the cytoplasm takes up the Romanowsky stains, the microgametocytes taking a very faint stain.

These figures were obtained in every case from observation of canaries which had been infected by blood inoculation; there is however great individual variation in different birds and the duration of the prepatent period is said to be greater when mosquito inoculation is used.

5. *Acute stage of infection.* In the case of *Plasmodium cathemerium* the crisis of the infection is generally reached three or four days after parasites first appear in the peripheral blood, and four or five days later they have nearly or quite disappeared. The acute stage of the infection produced by *Plasmodium inconstans* is usually considerably more prolonged, frequently lasting for two weeks or more, and it is seldom as severe. *Plasmodium præcox* is the most benign of the three. Parasites are often so few that, even at the crisis of the infection, it may be necessary to examine several oil-immersion fields to find any, and in this case it is the gametocytes which are most frequently seen in the peripheral blood. But although the infections are usually light they are very prolonged, and in many cases the crescent-shaped gametocytes of *Plasmodium præcox* can be found in the peripheral blood months after the acute stage of the infection has passed.

6. *Pathogenicity.* The three species also differ in pathogenicity. *Plasmodium cathemerium* is the most pathogenic of them all, the mortality averaging about thirty-three per cent during the acute stage. The death rate from infection with either of the other two parasites is almost negligible if the birds are healthy to begin with, and kept under favorable environmental conditions.

7. *Pathology.* The pathology of avian malaria is very similar to that of human malaria. The most marked changes are seen in the spleen which undergoes an enormous increase in size, and in the blood. The number of red cells drops very greatly during the course of the infection, and Ben Harel (1923) found that the mononuclears increased in number, and that the bone marrow showed a marked hyperplasia, with an accompanying decrease in the amount of adipose tissue present.

8. *Relapses.* When death does not occur during the acute stage the birds appear to recover completely, but except in rare cases the disease simply passes into a long period of latency, with occasional slight relapses which seem to be most frequent during the fall and winter, in the case of *Plasmodium cathemerium* at least (Manwell, 1929). Relapse is apparently less frequent about two

months after the original attack than either before or after this time, but there is much variation and certain birds are much more prone to relapse than others. Chronic infections with *Plasmodium cathemerium* are much less likely to relapse than are those of the other two species. Even though no parasites can be found in the blood of such a case for many months it is no indication that complete recovery has taken place.

9. *Other species.* In addition to the three species just described there is a fourth one, according to the Sergent brothers and Catanei (1928), for which they propose the name *Plasmodium rouxi.* No figures are published in their paper, but it is quite clear from their description that this species of parasite differs from those already discussed both in the type of infection which it causes, and in its morphology. Only four merozoites are produced by each schizont, instead of from twelve to twenty or even more as in the other three species, and the parasites are characterized by the presence of two grains of pigment of unequal size. The infections are always very prolonged, and the prepatent period is said to have an average duration of three weeks. Parasites can be easily demonstrated in the peripheral blood for a very long time, and the death rate is quite high; of fifty birds infected fifteen succumbed.

The "Whitmore strain," already referred to, is probably a variety of *Plasmodium inconstans.* This strain was originally obtained by Colonel Whitmore from a New York sparrow in 1913, and has been the subject of considerable work in the United States. It is very probable that there are still other species not yet recognized, for research in bird malaria has not been extensively carried on in the western hemisphere. There are also many species of *Hemoproteus,* a genus of blood parasites which is without doubt very closely allied to the plasmodia of birds.

10. *Source of parasites for experimental purposes.* Malarial parasites for experimental work are generally most easily obtained from sparrows, since these birds are usually available almost everywhere and at all seasons of the year. To determine whether a bird is infected a thin smear may be made and examined microscopically for parasites, but since very few parasites can usually be found during the latent period of the disease the better way is to inoculate a canary with 200 to 300 cubic millimeters of blood from the suspected bird. If parasites are present in the latter the

canary is virtually certain to show an acute infection within from two weeks to a month, depending on the species of *Plasmodium* concerned. Since the number of parasites is small during the chronic stage of the disease the length of the prepatent period in birds inoculated from latent cases will usually exceed the figures given above, although its blood should be examined for parasites four or five days after the initial inoculation and thereafter until they are found.

11. *Method of transferring infection.* The technique of transferring the infection is as follows: the large vein on the inside of the tarso-metatarsus is punctured with a small Hagedorn needle and the blood so obtained is sucked up into a small Luer syringe into which 50 or 100 cubic millimeters of citrated salt solution has previously been placed. (A solution containing two per cent sodium citrate and 0.7 % sodium chloride has been found very satisfactory for this purpose by the author. Physiological saline also works quite well but is less effective in preventing clotting.) When the requisite amount of blood has been obtained it is injected into a clean bird. It is usually found most convenient to inject the breast muscle, since there are no feathers in this region and those which are on the sides can easily be brushed away with a piece of cotton soaked in seventy per cent alcohol. A second advantage of inoculation at this point is the fact that there is usually little bleeding. If desired, intraperitoneal inoculation can be employed. There is less bleeding with this method than with any other, and with a little practice the technique is easily learned. Intravenous inoculation can be accomplished by using a No. 27 (or better, a No. 28 Luer needle. If the needle has been slightly bent by heating in a micro-burner, injection can be more easily made and there will be less danger of tearing the wall of the vein. The leg vein already referred to is the most convenient site for this type of inoculation, and will be found quite large in older birds. Intravenous injection will be found easier if the bird is first wrapped in a piece of cloth, in which holes have been left for the legs. The bird can then be placed on a table, with a small bit of cotton under the leg into which injection is to be made, and while the leg is held rigid with one hand the syringe is held in the other and the solution is forced into the vein *very slowly*. The process may be found somewhat easier if an assistant is on hand to hold the bird. Care must be used in

stopping the bleeding after the needle is withdrawn, not only to prevent the loss of too much blood but to avoid thrombosis. If the vein is pressed too tightly or too long, or if its wall has been much injured thrombosis is quite certain to occur. Aseptic precautions are not particularly necessary in work of this sort with birds, for they are very resistant to bacterial infection, nor do they seem to suffer much pain during the process. It is good practice to first clean all the areas with seventy per cent alcohol, however.

12. *Number of parasites necessary for infection.* The number of parasites required to infect a fresh canary varies with the weight of the bird, and with its natural resistance. Boyd (1925) found that if fewer than 1,000 parasites were injected infection seldom resulted, but more than this number were usually effective. When more than 10,000 were used inoculations were nearly always successful. In practice it is usually sufficient to take fifty cubic millimeters of blood from an active case, and less than this amount is often enough.

If for any reason a fresh bird cannot be immediately inoculated no harm will be done if the infective mixture is allowed to stand for several hours; the parasites appear to be very resistant to cooling and such a mixture has been found infective even after twelve hours. If placed on ice they remain alive much longer than this.

13. *Cultivation.* Although the parasites of human malaria have been successfully cultivated by Bass and others, those of bird malaria have not as yet been cultured outside the body of the host, although the same technique has been employed as for the parasites of man. It is likely that the chief reasons for lack of success lie in the necessarily small amounts of blood used. Whenever a simple and practical method of cultivating the avian parasites is devised the way will be opened for many experiments of the most fundamental importance.

14. *In vivo preparations.* Malarial parasites may be studied either *in vivo* or *in vitro*. If the living parasites are to be studied it is only necessary to mix a very small amount of blood from a well-infected bird with a drop of physiological saline, or citrated salt solution of similar tonicity, mount under a coverglass and observe with the microscope. Exflagellation can be very prettily observed under such circumstances, if gametocytes are abundant (the number of gametocytes reaches a maximum about one day

after the crisis of an acute infection). It will usually take place within fifteen minutes after the blood is drawn.

15. *Staining.* If a permanent preparation is to be made either Wright's stain or Giemsa's spirochete stain may be used. The former is the quicker, but in the writer's experience at least, the latter is more consistently satisfactory, and gives somewhat sharper stains. Such stains as hemotoxylin will not give satisfactory results with malarial parasites. Thick smears are not practicable both because of the excessive quantity of blood required, and because the nuclei of the red cells obscure any parasites which may be present.

The technique for making these stains follows:

a. Wright's stain

For Wright's stain the smear should be flooded for two minutes with the stain, after which an equal amount of distilled water is added and the mixture allowed to remain on the slide for four minutes more. It is then poured off and the preparation washed and allowed to dry.

b. Giemsa's stain

If Giemsa's stain is used the smear must first be fixed in methyl alcohol (absolute) for one or two minutes. It is then rinsed in running water and flooded with the stain, which is to be made up fresh each time it is used by taking one and one-half drops of the prepared stain and mixing it with one cubic centimeter of distilled water, up to whatever quantity may be required (from one to two cubic centimeters are required per slide). Twenty or thirty minutes are usually required to secure a good stain.

16. *Experimental work with drugs.* If experimental work designed to test the effects of various drugs upon the infection is to be done it is usually best to administer the drugs orally. This can be most easily done with a small Luer syringe of one cubic centimeter capacity or less, using a large size Luer needle from which the point has been cut for the purpose. The needle is then bent in such a way that the end can be conveniently thrust a little way down the throat of the bird, and it will be found that measured amounts of the solution can be given with very little regurgitation. It is of course important to see to it that no sharp edges remain on the cut end of the needle, or injury may be done to the bird's

throat. Compounds which are very toxic can often be given in greater amounts without poisoning the bird if the dose is fractionated. The time of treatment may also be of some importance since the parasites are assumed to be more vulnerable to the action of toxic drugs during the period of sporulation.

If desired, drugs can be quite easily administered by injection. Intramuscular and subcutaneous injections can be most readily performed in the region of the breast because of the scarcity of feathers in this part of the body. The method of making an intramuscular injection into the breast muscle has already been described. Intraperitoneal injection is almost as convenient, and intravenous injection can be done if desired. The technique for this has already been given. It is of course always essential to see to it that the solutions injected are isotonic with the blood, and it is also well to have them warmed to about 40° C.

17. *Measuring the effect of drugs.* The effect of drugs or of other agents upon the course of the infection may be measured in various ways, depending somewhat on when the treatments are given. The author has always believed that the effect could best be gauged by changes in the length of the prepatent period. If this is to be the index of success, the treatment must be commenced very soon after the initial inoculation of the parasites, and daily blood smears must be made until these are found. Any substance which tends to lengthen the prepatent period may be assumed to exert a curative effect, and vice-versa.

If the drug is not administered until parasites can be demonstrated in the blood then its effect can be measured either by making daily counts of the number of parasites per unit number of red cells (in practice 10,000 has been found a very convenient unit), or by making polar planimeter determinations of the areas of the parasites at different periods during the course of the treatment. The latter method was devised by Hartman (unpublished report). This worker (*1927*) found however that as the number of parasites increased a progressive decrease could be noted in their size, quite irrespective of any treatment, so that this factor must be taken into account. It is not very practical to make daily counts of the red cells, or of the parasites contained in them, because the amount of blood in a normal canary bird is not much greater than one cubic centimeter and during the course of a malarial infection there is a very severe anemia. It is of course still less practicable to make leucocyte counts, although their ratio to the number of red blood cells and differential counts are easily obtained. Since most birds show no symptoms of illness until they are really very sick it is impossible to tell much as to the effect of any treatment by direct observation of the bird itself.

18. *Quinine and plasmochin.* At the present time there are only two known drugs which have been shown to exert any effect on the bird malarias. One of these is quinine and the other is plasmochin. When the former is administered in doses of about one and one-half milligrams per day it will always prolong the prepatent period, and will very often entirely prevent the appearance of parasites, although such birds continue to carry a chronic infection thereafter just as if they had recovered normally. Since the M.L.D. for quinine is about six milligrams for a bird of average weight (canaries vary in weight from twelve to nineteen or twenty grams, with a mode of about sixteen and one-half grams) it is not practicable to increase the dose very much, the more especially since the drug has a cumulative effect. Plasmochin is a vastly more effective drug than quinine, as far as bird malaria is concerned. It is also much more toxic, but its therapeutic power is so much greater that the doses required are relatively less toxic. The M.L.D. is about one and one-half milligrams for a bird of average weight, but 0.1 milligram administered daily for four or five days is enough to entirely prevent the appearance of parasites (although the blood of such birds remains infective, just as with quinine). Roehl (1926) found that doses as small as 0.02 of a milligram were effective when given daily. The value of these two drugs has been discussed in some detail in order to make it clear as to what may be expected of a really effective therapeutic agent.

PROBLEMS FOR FURTHER INVESTIGATION

1. *Drug therapy.* Although much work has been done on malaria, both in birds and man, there is great need for further research, not alone to discover new facts, but to verify experimentally certain beliefs which have long been held without any very definite evidence in their favor. The results already obtained with plasmochin show clearly the practical value of research in the field of drug therapy, and we need especially to know exactly how such drugs exert their beneficial effects. Do they act directly on

the parasite, or is their effect an indirect one due to some interaction of drug and tissues of the host?

2. *Biology of the parasite.* There are still many things which we do not know about the parasite. We need information about its cytology, its life processes, the mechanism and details of gametocyte formation, the question of whether or not a toxin is produced, and the number of species which exist. There are many facts which a careful study of pure-line infections might bring to light, and an entire new field of research would be opened up if we could cultivate the malarial parasite of birds (or of man) as we do bacteria.

3. *Host-parasite relations.* Similarly there are many things which we do not yet know about the disease in the host, and the reaction of the latter to the parasite. We do not know whether the injury which the latter does the host is confined to the destruction of the red blood cells or not, and, although we have good reasons for supposing that this is not the sole mode of injury, we have little data as to what these other possible modes of injury may be.

4. *Relapse.* It is unnecessary to remark that the problem of relapse is one of the most important in the whole field of malaria, and it is likewise a phase of the disease about which we know much less than we ought. We have little information as to the mechanism of the resistance which the host develops towards the parasite, and still less as to why it is that certain mosquitoes make better intermediate hosts than others do. We have as yet no reliable method for the quick diagnosis of carriers, either avian or human, and we are quite ignorant of just what changes in the host (or in the parasite) may be responsible for the occurrence of relapses.

5. *Geographical distribution.* In the case of bird malaria we have not the slightest information as to the geographical distribution of the different species of parasite. We know that they are collectively very widespread it is true, but of exact knowledge we have next to none. We also know relatively little as to the particular species of *Culex* or *Stegomyia* which act as vectors in different regions.

SPECIFIC PROBLEMS FOR INVESTIGATION

A. Drug Therapy. It would be desirable to find:

1. A drug which would sterilize an infected bird completely, for such a drug would probably be of value in treating human malaria.
2. Drugs which would have a beneficial effect on the disease, even though this effect fell short of sterilization. Such drugs would be likely to add to the effectiveness of present methods of treatment.
3. Substances which would decrease the proportion of asexual forms to gametocytes. If such an effect could be made marked enough it would affect the course of the disease very favorably as far as the individual was concerned, and isolation would safeguard the health of others.
4. Compounds which would act selectively on the gametocytes. Such drugs would be likely to be valuable in the treatment of human malaria, for if they exerted an effect of this kind it would help solve the carrier problem.
5. Exactly how the useful compounds which we already have exert their beneficial effect. Do they act upon the host or upon the parasite, or do they act in both ways? And in either case, to just what is the effect due? *e.g.,* Is the rate of oxidation of the parasite altered? Are the red cells made impermeable to the young merozoites after liberation from the previous host cell?
6. At exactly what stage in the asexual portion of the life cycle are the parasites most vulnerable to the effective drugs which we now have?
7. A drug which when administered to the patient would render the sexual portion of the life cycle impossible of development in the body of a mosquito subsequently biting the patient.
8. By bird experimentation, whether there is any difference in the efficacy of a drug when administered by varying routes.
9. Whether either quinine or plasmochin ever stir up relapses in latent cases of avian malaria, as salvarsan is said to in human malaria, by the production of a kind of a Herxheimer reaction.
10. To try salvarsan and its near relatives on the four known species of bird malaria, to see whether it exerted any effect.

(It has already been tried on bird malaria, but it is impossible to tell what species of parasite was concerned.)

11. To make a comparative study of the effect of quinine and plasmochin on the different species of avian parasite, and determine whether all these species are equally affected. (Since there are known to be such differences between the human malarias.)
12. Whether the effect of quinine and plasmochin can be laid to any particular part of the molecule. (Much work has already been done on this point but without conclusive results.)
13. Whether, after large doses of plasmochin, infective birds are any less infective to mosquitoes biting them within the next two or three hours. (It has been found that quinine is without such effect.)
14. Whether plasmochin exerts any selective action on the gametocytes of any of the species of bird malaria, as it is reputed to do in the case of *Plasmodium falciparum*.
15. Whether prolonged treatment with plasmochin would ever result in the complete sterilization of the treated bird.
16. What effect, if any, plasmochin exerts on the parasites of avian malaria *in vitro*.
17. Is there any difference in degree of infectivity between the blood of a plasmochin-treated case, and a case which has spontaneously recovered?

B. The Parasite:

18. What the geographical distribution is of the species now known. (Is there any grading into one another, as might be expected if there is a close evolutionary relationship?)
19. What the seasonal prevalence of infection with the different species is.
20. Whether cross-fertilization between gametocytes of the same species is possible in the body of the mosquito.
21. Whether there are actually periodical changes in virulence, such as it is likely exist in human malaria, and which the work of Manwell suggests may exist in bird malaria.
22. Whether continued asexual multiplication has any effect upon the vitality of any of the known species.
23. Whether the parasites produce a toxin of any sort, and if so, what the nature of this toxin is.

24. Whether the changes which take place in the proportions of asexual forms to gametocytes during the course of the disease are due to (a) selective destruction by the host, or (b) whether there is actually a greater production of gametocytes later in the infection.
25. Whether gametocytes ever develop directly from sporozoites.
26. Whether, if the proportion of gametocytes developed later in the disease is really greater than that produced earlier, the change is due to the increasing resistance of the host. (This hypothesis might perhaps be tested by making comparisons of the changes in the asexual parasite-gametocyte ratio in birds showing light infections, and in those showing long infections ending in death. For it might be assumed that in the latter case resistance was not increasing in the host as much as in the former.)
27. The cytology of the parasite, with respect to chromosome number, etc., ought to be investigated, both in the asexual portion of the cycle and in the mosquito. (But this cannot be done successfully until a better method of staining is devised.)
28. Why some mosquitoes afford suitable conditions for the development of the parasite when others do not.
29. Whether development, or any part of it, can occur in the body of any other biting insect.
30. The effect of various environmental agents, such as ultraviolet light, upon the parasite *in vitro*. (Investigation of such changes should include not only the visible ones, but changes in virulence, etc. And changes in the chromosome number ought to be looked for.)
31. A practical method of culturing the parasite outside the body of the host.
32. How pure-line infections (those started from a single parasite) would compare with ordinary ones.
33. A method of producing pure-line infections. This would not be difficult if cultivation were possible. It might be possible to secure a pure-line infection by infecting a bird with the sporozoites from a single oöcyst of an infected mosquito. It is said that as many as 10,000 sporozoites may be developed from such an oöcyst, and this would probably suffice for infection.

34. How mosquito-caused infections compare with infections by blood inoculation. (The earlier workers believed that there was a definite difference, and there appears to be such a difference in human malarial infections which are induced by blood inoculation.)
35. Exactly what the requirements of the parasite are as to oxygen consumption, anaërobiasis, glucose consumption, etc. (Such information might pave the way for successful cultivation.)
36. Whether other animals can be infected with the avian malarial parasites. (Koch made a beginning in this field, when he made an unsuccessful attempt to infect a monkey.)
37. Whether blood serum is toxic to the merozoites as has been claimed.
38. Whether there are other species of bird malaria.
39. Whether changes can be induced in the parasite by exposure to various agents while in the mosquito. (Since the sexual stage in the life history takes place in the intermediate host it would seem that such changes could be more easily induced then. Radiation of different sorts would be a desirable agent to experiment with.)
40. A critical and careful study of infections produced before and after mosquito passage might be of considerable biological interest.
41. Whether the parasites are experimentally infective to birds at any stage before the sporozoites are liberated from the oöcyst.
42. What constitutes resistance to infection in the mosquito. (It might be possible to test whether such resistance was heritable by experimental means.)
43. Whether the proportion of gametocytes varies with the season, as has been suggested by Ross and others, and indicated by the work of Manwell, 1929. It should be determined whether, if such a variation really occurs, it is a real variation in the proportion of gametocytes to asexual forms, or whether it is only an apparent variation, due to the greater severity of relapses in the seasons in question.
44. The length of the asexual cycle in *Plasmodium inconstans* and *Plasmodium præcox*.
45. Are there any other measurable or detectable differences be-

tween the oöcysts of various species than those already reported in size (Huff, 1927)?

46. Whether the proportion of male to female gametocytes can be changed in any way.
47. Whether this proportion is noticeably variable in different cases, and whether it is approximately the same or significantly different in different species.
48. Acton and Knowles (1914) believed they found clear evidences of parthenogenetic development of the macrogametocytes *in vitro* in the case of the halteridium parasite of the pigeon. Does this ever happen in any of the species of bird malaria?
49. Whether the number of parasites necessary to produce an infection is different in the different species.
50. How many sporozoites of the different species are necessary to bring about infection.
51. What the length of life of gametocytes is.
52. Whether the male and female gametocytes are equally resistant to various agents.

C. The Host:

53. Where in the body of the host are parasites chiefly destroyed?
54. To what is the great congestion in the spleen due?
55. Are gametocytes destroyed chiefly in the spleen, as some believe?
56. What effect does malnourishment have on the resistance of the host to malarial infection?
57. What is the effect of fasting in inducing relapse? (Casagrandi believed it an important etiological factor.)
58. What effect would lack of vitamines have on the resistance of the host?
59. What features of the environment are most important in (a) the production of relapse, and (b) favorably affecting the course of the disease?
60. In what part of the body of the host does segmentation chiefly occur in the case of *Plasmodium præcox?*
61. How do the different species compare with respect to the liability to relapse as the time after the original infection increases?
62. Is this increase in the frequency of relapse (which has been

shown to exist in cases infected with *Plasmodium cathemerium*) merely a function of the increasing age of the bird?

63. Is there any "age resistance" on the part of the host to malarial infection? (Entirely apart from any possible immunity resulting from infection in early life.)
64. Just how soon, under ordinary conditions, does the blood of a newly infected case become infective?
65. Can a method be devised for the quick diagnosis of latent infections (as by serum test)?
66. How often does complete recovery take place in untreated infections with the different species?
67. Exactly how do the parasites injure the host (other than by blood cell destruction)?
68. Do the various agents which will produce relapse have anything in common? (*i.e.,* Do they produce the same effect on the host, or is there something fundamentally similar in the agents themselves?)
69. What is the mechanism of resistance to malarial parasites?
70. Does resistance to superinfection in the case of a bird carrying a chronic infection mean that no amount of superinfection will produce an acute attack?
71. What effect do physical agents, such as light of various sorts, heat, excessive cold, humidity, barometric pressure, etc., have on the resistance of the host?
72. How is this effect exerted, and do differing agents produce the same effect on the host in the same or different ways?
73. What measurable or perceptible changes does the disease produce in the host—*e.g.,* changes in metabolism, etc.?
74. Splenectomy has been tried in human malaria. What effect would it have on avian malaria?
75. What factors are responsible for the definite length of the cycles in the case of the parasite, and are these inherent in the host, or in the parasite (or, more probably, to some extent in both)?
76. Whether relapses are primarily due to changes in the resistance of the host (as seems most likely) or whether there are changes in the virulence of the parasite.

CHAPTER XXXVIII

PERIODICITY IN MALARIA

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Various methods may be used to study the periodicity of the asexual cycle of malarial parasites. A very accurate method involves a statistical consideration of the changes in size of the parasites such as has been recently used in the malarial parasite of birds (L. G. Taliaferro, 1925). This method does not differ in principle from a simple inspection of the slides, but it eliminates to a large degree the unconscious selection of forms and gives a numerical basis for comparisons of the average size at different times. Another valid method involves ascertaining the ratio of sporulating individuals to the total parasite population which has also recently been used in a study of the avian parasite (Boyd, 1929). Where parasites are scarce, the first method will probably prove more serviceable; where parasites are numerous, the second method will probably be simpler.

If either of these methods could be applied to the human malarial forms, they might elucidate some of the moot questions. Inasmuch as both methods depend upon the occurrence of all of the stages in the peripheral blood, *Plasmodium falciparum* could not be as readily studied because the young merozoites leave the peripheral circulation early in their development to complete it in the internal organisms. Nevertheless, a number of interesting facts might be obtained by modifying the first method and studying the growth of the parasites when they did occur in the blood, together with the time of their appearance and changes in number. Moreover, cultural forms might be studied, although whether or not they would be comparable to the *in vivo* developmental forms is not known. For example, Perekropoff (1914) studied *P. falci-*

parum in cultures at 41° C. for five generations and concluded that the cycle was probably of twenty-seven hours' duration.

Each of the methods, with its various applications, will be considered separately.

The procedure in the first method, which has been fully described in a previous paper (L. G. Taliaferro, 1925), consists in obtaining the mean size of representative samples of asexual forms at frequent intervals through as much of an infection as is possible and may be outlined briefly as follows:

1. Thin[1] blood smears are made every two or four hours through the day and night over at least several days or longer if possible. These are stained with Wright's stain.

2. From slides from each time-interval 50 or 100 outline drawings of the asexual forms[2] as they are found at random are made at a standard magnification by means of a camera lucida. (x3000 seemed a convenient drawing magnification.)

3. The drawings from each set are measured by obtaining the product of the average length by average width in some convenient standard. (Microns may be used.)[3]

4. The mean $\frac{(\Sigma x)}{n}$ of each set is obtained by dividing the sum of the measurements (Σx) by the number of individuals drawn (n).

When these data are plotted graphically against the time of an infection, they will give, where there is a periodicity, a curve somewhat like that shown in Fig. 26 (curve of the mean size).

Such a curve, since it is based on averages, does not indicate the degree of synchronicity of the cycle. This may be ascertained by making frequent slides during the time of sporulation and finding the percentage of young forms. If synchronicity were absolute, at a given instant, the percentage of young forms would increase from 0 to 100%. If, on the other hand, there were no synchronicity, the percentage of young forms would remain constant throughout the infection. Such a procedure will also disclose whether or not an infection is simple or multiple, for where there is more than one brood of parasites, the percentage of very small forms will never reach the high percentage attained in single infections, such as is shown in Fig. 27.

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[1] Thick smears might also be adapted for use which would enable the study of lighter infections.

[2] It was found best during the course of the work to omit the gametocytes as they mask, to some extent, the changes in mean size of the asexual forms.

[3] This measure of "size" is neither a measure of volume nor cross section, but is easy to obtain and sufficiently accurate.

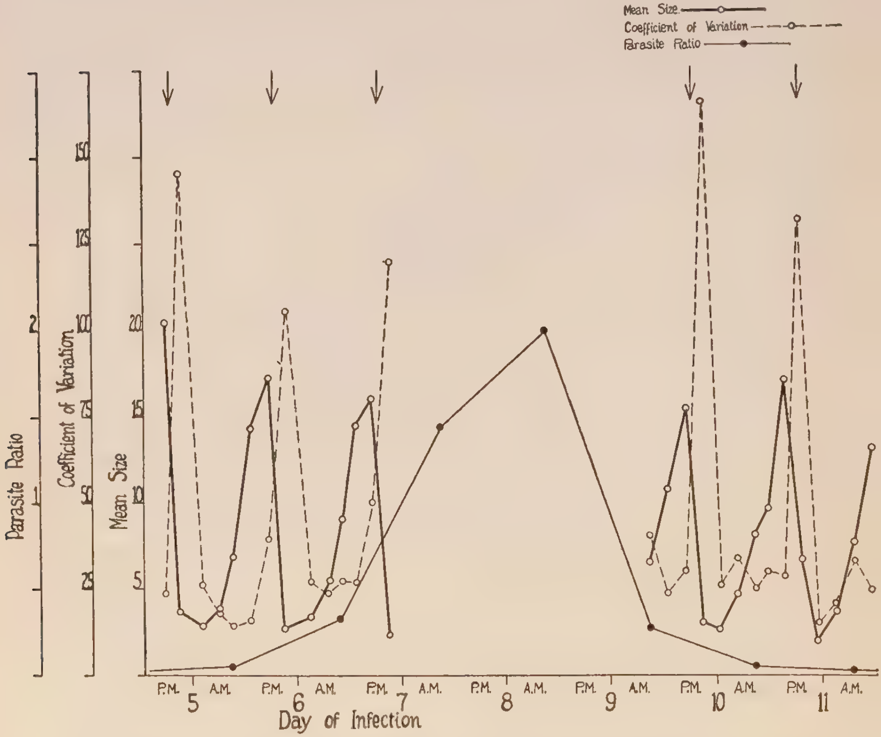

FIG. 26.—Graph showing the changes in mean size, variability in size and number counts (ratio of parasites per 10,000 red blood cells) for the asexual forms through the acute and beginning of chronic periods in Bird 61. The arrows indicate the time of maximum sporulation. (From L. G. Taliaferro, 1925.)

Some additional data will indicate the extent of the synchronicity and consist in ascertaining the coefficient of variation for size of the parasites from the data already obtained. Briefly, the coefficient of variation may be found as follows:

1. The standard deviation is calculated from the measurements already obtained by the formula $\sqrt{\frac{\Sigma x^2}{n} - M^2}$, in which Σx^2 represents the sum of the squares of the individual measurements them-

selves (not their deviations, as is ordinarily used), n, the number of parasites drawn and M^2, the square of the mean.

2. The coefficient of variation is derived from the formula $\frac{100\sigma}{M}$, in which σ represents the standard deviation, and M, the mean. These data will indicate the sharpness of the periodicity. For example, if the cycle is fairly synchronous, the coefficient of variation will be low at all times (there will be little variation in size), except at sporulation time (when there will be both large and small forms).

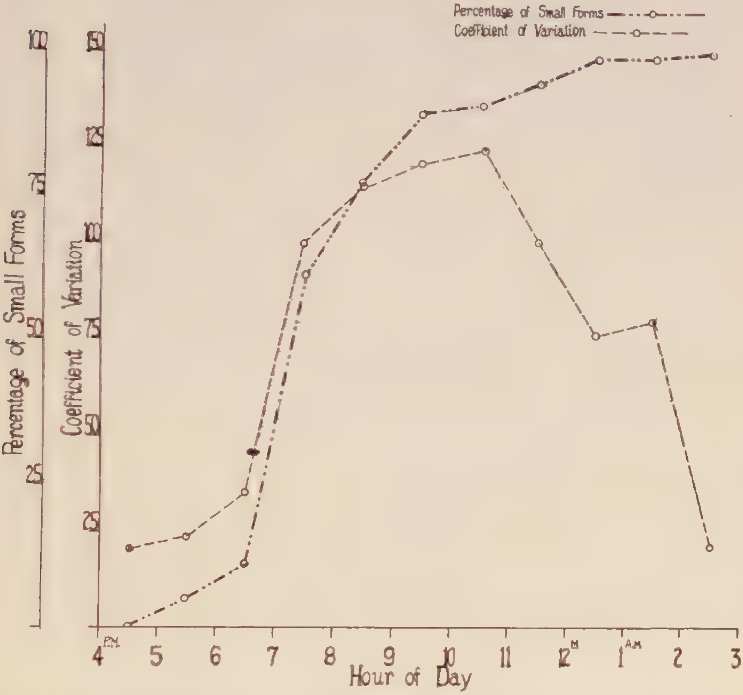

FIG. 27.—Graph showing percentage distribution of small forms and coefficient of variation for all forms during one period of sporulation in Bird 40. (From L. G. Taliaferro, 1925.)

Applications of these methods based on changes in size have brought out some interesting facts in avian malaria. The present author (1925) has found that in one strain, *Plasmodium cathemerium* Hartman, 1927*b* (= *P. præcox* or *relictum* of earlier work), the cycle was twenty-four hours [4] throughout the acute, crisis, chronic and relapse periods, in fact whenever parasites could be found in the blood. These facts are illustrated graphically in Fig. 26 which shows the changes in mean size of the asexual forms encountered in Bird 61 through a part of the acute and chronic period. The synchronicity of the strain is shown by the coefficient of variation curve (Fig. 26) which is fairly constant and low during the major portion of the cycle (varied roughly between fifteen and thirty-five per cent) but rises and falls precipitously at each sporulation-time (100-135 per cent). The duration of sporulation is shown in Fig. 27 where the percentage of small forms and the coefficient of variation are plotted from data at hourly periods during one period of sporulation and indicate that whereas most of the parasites sporulate between 6:30 and 9:30 P.M., there are a few stragglers before and afterward. The cycle in this strain is, therefore, fairly synchronous. Roughly, at 10:00 P.M., all of the asexual forms are small in size, they then grow in size until by 2:00 P.M. the next day they are all large, and at 6:00 P.M., large and small forms, representing sporulation, both occur (Fig. 28). As has been previously pointed out, where the cycle is simple and growth of the forms is nearly synchronous, these changes can be roughly gauged by inspection of the slides, but where there is any marked lack of synchronicity, the observer is liable unconsciously to be biased in his selection of forms.

In another strain of avian malaria, isolated by Whitmore and which Hartman (1927*b*) has identified as *P. præcox* (Grassi and Feletti, 1890), the author found a thirty-hour cycle. This strain is not as synchronous as the one just described; the coefficient of variation curve is higher during the non-sporulating periods (varied roughly between forty and seventy per cent) and did not rise as precipitously nor as high (81-104%) during the sporulation periods. The cycle, moreover, is not as clear cut and the following facts make it desirable that the work be repeated: The sexual as well as the asexual forms were included in the mean size curve, whereas it would probably be preferable to omit the sexual forms in view of the fact that Hartman (1927*b*) stated that the asexual forms tended to disappear from the peripheral blood as they do in *P. falciparum*. Hartman also stated that the cycle was approximately twenty-four hours, unless, as seemed un-

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[4] After a three months' study of the cycle I concluded that it was twenty-four hours and one minute, but with longer studies, Hartman (1927*a*) and I (1928) have found it to be exactly twenty-four hours. The occurrence of the cycle has been corroborated by Drensky and Hegner (1926) and Boyd (1929).

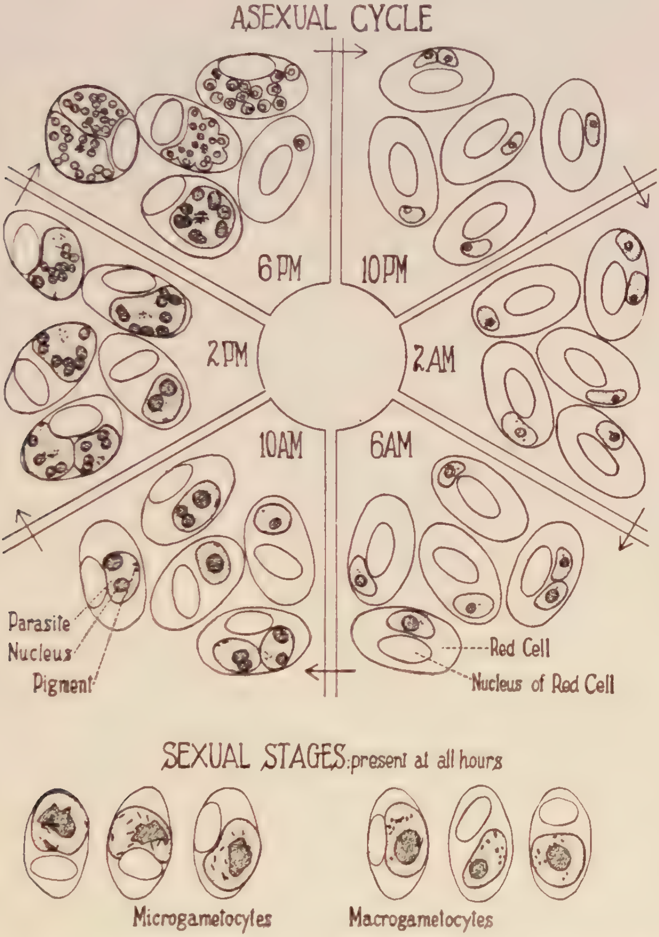
ASEXUAL CYCLE
6PM
10PM
2AM
6AM
10AM
2PM
Parasite
Nucleus
Pigment
Red Cell
Nucleus of Red Cell
SEXUAL STAGES: present at all hours
Microgametocytes
Macrogametocytes

FIG. 28.—Representation of the cycle of reproduction in bird malaria showing changes in size. Outlines of the asexual stages of the parasites within the nucleated red cells, showing nuclei and pigment granules, made at four-hour intervals during a consecutive period of twenty-four hours. In addition, outlines of three microgametocytes and three macrogametocytes, which occur in small numbers at all hours throughout the infection. × 1500. (From L. G. Taliaferro, 1928.)

likely to him, there was a double infection with two broods of parasites, each with a forty-eight-hour cycle. The strain has been kept by direct subinoculation in canaries for over eleven years and this may have altered the synchronicity even if the basic time of the asexual cycle remained unchanged.

With the cycle and its periodicity established in avian malaria, the next question appeared to be how constant is the cycle. Recent data indicate that it is remarkably constant and correspondingly difficult to change. By refrigerating parasites (*P. cathemerium*) for six, twelve, and eighteen hours and by injecting very large numbers into birds intravenously so that studies could be made from the moment of injection, the present author (1928) found that the cycle was delayed by the refrigeration, but that it completed itself in approximately twenty to twenty-two hours, instead of the customary twenty-four hours, until sporulation again occurred at the regular time from which it did not thereafter vary.

The foregoing work has dealt with the periodicity in asexual forms. Recently, Cowdry and Cowell (1928) have studied blood smears made at four-hour intervals over a three-day period from a monkey infected with *Plasmodium kochi.* They found that the size of the male and female gametocytes varies more or less synchronously during approximately a twenty-four-hour cycle which reaches its peak at about 4:00 P.M., and that the females are generally slightly larger and more numerous.

The study of the cycle has also yielded interesting results in relation to the acquired resistance of the body to malarial infections. Although it has often been tacitly assumed that an acquired resistance resulting in a decrease in the number of parasites is associated with a decrease in the *rate* at which the parasites are reproducing, W. H. Taliaferro and the present author (1922) pointed out that a decrease in numbers signifies a destruction of the parasites which may or may not be associated with a decrease in the rate of reproduction *per se,* and that the two factors must, therefore, be differentiated in any analysis of an acquired resistance. Such a study has been undertaken in avian malaria by making frequent number counts and by comparing the length of the asexual cycle in the various parts of an infection when the parasites are present. Parenthetically, it may be said that the length of the asexual cycle which is a direct measure of the time it takes for a small merozoite to produce approximately sixteen (in avian malaria) merozoites[5] is indepenent of the number of parasites destroyed. Thus, the rate of reproduction was found to be constant in *B. cathemerium*, since the cycle took twenty-four hours throughout the acute, crisis, chronic and relapse periods (in fact, whenever parasites could be found in the blood), whereas the number of parasites decreased remarkably at the crisis and subsequently. (See Fig. 26 for data during the acute and beginning of chronic periods.) Hence, it could be concluded that the bird develops, in the course of its infection, a parasiticidal resistance but no resistance affecting the rate of reproduction of the parasites.

The second method, which has been fully described by Boyd (1929), consists in making blood smears every two or four hours through the day and night, staining them with MacNeal's tetrachrome stain, and in determining the percentage of sporulating forms to the total parasite population. The method is discussed in more detail in Chapter XXXIX.

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[5] The number of new merozoites produced is constant for the various stages in the infection (L. G. Taliaferro, 1925).

CHAPTER XXXIX

EXPERIMENTAL MODIFICATION OF BIRD MALARIA INFECTIONS

I. BY CHANGING THE SUGAR CONTENT OF THE BLOOD

By

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II. BY CHANGE IN ENVIRONMENT

By

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I. CHANGING THE SUGAR CONTENT OF THE BLOOD

Preliminary statement. Bass (1912) in his effort to cultivate the organism of human malaria on artificial media, and others who have repeated his work, found that the addition of sugar to the culture medium was necessary for success. Bass and Johns (1913) reported the cultivation of the organism of human malaria in the blood of a diabetic without the addition of sugar. It has also been found that certain provocatives which bring on relapse in bird malaria, *e.g.*, epinephrin, exposure, etc., increase the sugar content of the blood. In an effort to study relapse (Hegner and MacDougall, 1926), it seemed worth while to observe the effects of changing the sugar content of the blood of the birds, especially during the period of sporulation.

Materials and methods. Infection with the Hartman strain of bird malaria, *plasmodium cathemerium,* was transferred from one female canary to another by direct blood inoculation, injections of about 0.05 of a cubic centimeter of a normal saline dilution of infected blood being made in the peritoneal cavity. The blood was obtained from a vein on the inside of the leg. An effort was made to eliminate environmental and other conditions which might affect the normal course of infection. Riddle and Honeywell (1923) found that blood sugar in pigeons changes with exposure, sex, ovulation, etc., and Boyd (1925) suggested that excessive bleeding might account, in part, for the variations in the normal course of infections observed by other workers. The work reported here seems to confirm this suggestion. Birds bled for quantitative work very frequently show heavy infections.

It seemed that the simplest way to increase the sugar content of the blood is to feed birds a solution of glucose. Ten grams of glucose were dissolved in ten cubic centimeters of water, and the birds fed four times a day at 3:00, 5:00, 7:00 and 9:00 P.M. This covers most of the period of merozoite formation when the parasites might be expected to be most easily affected by a change in the culture medium (the blood). It was found that changing the sugar content of the blood at any time except during the time of merozoite formation had little, if any, effect on the course of bird malaria. The birds seemed to thrive on it. A dose of one-tenth of one cubic centimeter was administered on the first day of the feeding of glucose at each of the four hours mentioned, then increased from 0.5 to 1 cubic centimeter at each feeding as the birds grew fond of it. They would take it even when sick.

The method used to decrease the sugar content of the blood was to inject insulin intraperitoneally. It was found that the maximum dose for most birds was two units. In several cases where three units were administered, one dose at eleven o'clock, one at three, and one at six, the birds were unable to stand. Prompt recovery in a few minutes followed the feeding of dextrose. Quantitative work has shown that birds vary a great deal with respect to normal blood sugar. It is to be expected, then, that the insulin should be more effective in some cases than in others.

Suggested problems. Quantitative work is necessary to show the relation of the blood sugar to the normal course of malaria, relapses, etc. Much work along this line has been done with canary blood, but, owing to the very small amounts of blood obtainable from the birds, it would seem that this work must be done in cases of human malaria where conditions can be controlled and where there is a sufficient amount of blood to be sure of the results. On account of the large experimental error due to the small amount of blood obtainable, the data with regard to quantitative work on the canary blood have not been published.

In the one human case followed, it was shown that the blood sugar increased during the paroxysms of chill, etc. This suggests that at least twenty-five cases should be accurately followed, the blood sugar being determined before inoculation with malaria, and determinations being made between and during paroxysms of sporulation. In most cases where malaria is used as an agent for the treatment for disease, a heavy infection is wanted, so it would do no harm to administer glucose during the period of sporulation of the parasite if that period can be determined. It will do no good to administer it at any other time.

II. CHANGES OF ENVIRONMENT

That infections of canaries with *Plasmodium cathemerium* are characterized by a definite periodicity in the reproductive activity of the parasite was shown by L. G. Taliaferro (1925). She showed that pronounced periods of reproduction occur at twenty-four-hour intervals, and her observations in this connection have been confirmed by Drensky and Hegner (1926) and Hartman (1927). These infections of birds, therefore, offer suitable material for the study of this interesting and important phenomenon which is such an outstanding feature of certain malaria infections of the human.

A common explanation of the above-mentioned periodicity in human malaria is that each species of parasite in these infections has a definite length of its asexual cycle of development. Since such is the case and since all parasites injected into the vertebrate host by the mosquito are in the same stage of development (namely, the sporozoite), it is assumed that they undergo further development synchronously and reach maturity and enter upon the process of schizogony at approximately the same time. The continuation of such synchronism results in the subsequent repetition of these periods of reproduction at regular intervals. Based upon such an explanation, the idea has become very prevalent that the basis for the phenomenon of periodicity in malaria is a matter which is firmly fixed in the inherent make-up of the causative organism and little influenced by conditions which may surround the host or the parasite.

Protozoa in general do not seem to follow such regularity in the matter of reproduction and this fact leads one to question the above-mentioned assumptions in regard to the cause of periodicity in malaria. Accordingly, experiments were undertaken by the writer (see Boyd, 1929*a* and 1929*b*) to determine whether the reproductive activity of these parasites is definitely fixed or can be modified by alterations of the environmental influences which usually surround these infections. These experiments may be grouped under the following heads:

1. *Reversal of the daily cycle of activity and rest for infected birds.*
2. *Simultaneous inoculation with two groups of parasites differing in their periodicity—that is, so-called double infections.*
3. *Variation in length of the experimental day and night for infected birds.*
4. *Continued exposure of infected birds to an experimental day and night of fourteen hours each.*

Material and general methods of procedure. The parasites used were from a strain obtained by Dr. Ernest Hartman from an English sparrow in Baltimore, Maryland, in 1924, and later described by him under the name of *Plasmodium cathemerium.* Female canaries were used as the experimental host in these studies. The infection was transmitted to these canaries by direct inoculation with a small number of parasites from a previously infected bird. In the effort to control experimentally the cycle of daily changes which normally go on in infected birds and their parasites an attempt was made to simulate day and night in an artificial way by shifting these infected birds alternately at chosen intervals between a room which was lighted by means of an arc lamp giving approximately 3000 candle power illumination and a dark, unheated closet.

In order to follow the reproductive activity of the parasites under the experimental conditions, blood smears were taken from the infected birds at intervals of two hours throughout the period of study. These smears were stained with MacNeal's tetrachrome stain. The tetrachrome stain was used in preference to others because of the fact that it gives particularly definitive results in the staining of segmenting forms and it stains with a readiness which is to be desired in such work. The degree of reproductive activity of the parasites for any given time was determined by the direct method of counting the number of asexual forms of the parasite in a succession of microscopic fields and noting particularly the ratio of segmenting forms to this total number of the parasite population. Only those forms in which the process of schizogony had proceeded to the point that the cytoplasm of the parent individual had actually begun to divide to form merozoites were included as segmenting individuals. Obviously the validity of our observations is dependent upon the number of parasites that were included in our counts, or the size of the sample upon which the observations were made, and the size of sample used for observation was necessarily greater when reproducing individuals were scarce than when they were plentiful. The general rule which we have followed in these studies was that of making our observations upon samples of sufficient size to reduce the probable error to fifteen per cent of the total observed values. Often it was ten per cent, or less, but when little or no reproduction was taking place it was usually unnecessary, for our purposes, to maintain such a high degree of accuracy. Even under these conditions, however, the size of the sample was always such as to insure a relatively low probability of error.

CHAPTER XL

SEROLOGICAL METHODS IN THE STUDY OF THE PROTOZOA

By

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INTRODUCTION

A. Limitation of the subject. Fundamentally, the science of immunity in protozoan infections is concerned with the resistance of the body to infection and disease. Following the general trend of the development of the broader science, however, work with the protozoa has, to a large extent, dealt with serological (test tube) reactions because of their practical value. Although a great deal of work has been carried out on the symptoms in the protozoan diseases, the production of toxin and toxin-antitoxin reactions, the hypersensitive states and anaphylaxis, cutaneous tests, with especial reference to the detection of protozoan infections, and the nature and artificial production of immunity, by far the greatest amount of work has been focused on the serological reactions.

In the present review, therefore, attention will be limited to the serological reactions—precipitation, agglutination, lysis, complement fixation and certain miscellaneous reactions of the serum. These will be considered from the standpoint of their use as test tube reactions, and, where applicable, of the part they assume in the defense of the body against infections with parasites. In the latter case, their relation to the reproduction-inhibiting antibody, demonstrated in certain trypanosome infections, will be discussed. Since it would be impossible to review the mass of specific investigations, attention will be particularly centered on technique and the more important investigations. For the general subject of immunology the reader is referred to the standard treatises on immunology. Of those in English, particular attention is due Kolmer (1923 and 1928) for technical details; Zinsser (1923) for general discussions; Wells (1925) for the chemical aspects; and the compendium of Jordan and Falk (1928) for a critical discussion of many specific immunological questions by various authors. For a special consideration of the immunology of the parasites and an extensive bibliography, the reader is referred to a book by the author (1929).

B. General nature of antigens, test antigens, antibodies and serological reactions. The parenteral introduction of a large class of substances, termed antigens, into an animal is followed after a lapse of time by the appearance of antibodies in the animal's serum which in turn will react specifically, not only with their specific antigens but with certain derivatives of them in *in vitro* tests. In order to include all the substances which will react with antibodies in the serological tests and to differentiate them from true antigens, the term "test antigen" has been used in the present review. More explicitly, substances to be antigens, *i.e.,* stimulate the production of specific antibodies in an animal, must be in colloidal solution, must be foreign to the test animal, must penetrate beyond the epithelial surfaces which protect the body, most probably must be protein (Wells, 1925), and may be infecting organisms or their derivatives (including toxins) or an inexhaustible number of proteins, such as egg albumen, blood, etc. Substances to be test antigens, on the other hand, *i.e.,* able to react specifically with serums of animals in which the antibodies occur as a result of immunization or infection, may be protein, but are not necessarily so. Thus, in some cases, they are identical with the true antigens and are protein, as when highly purified proteins, such as egg albumen, are used. In other cases, complex carbohydrates (Zinsser's "residue antigens," Avery and Heidelberger's "specific soluble substances") or lipoids (Landsteiner's "haptenes") stimulate the production of antibodies only if they are in combination with proteins, but later, can react by themselves specifically with the resulting antibodies and with even more precision than the corresponding proteins alone.

Tremendous strides have been made toward an exact chemical understanding of the nature of antigens and test antigens, but little so far is known of the nature of antibodies. They have not been isolated chemically, but are known as properties of serums (hence, antiserums) and are postulated in terms of what they do rather than what they are (precipitins, agglutinins, etc.). Some immunologists believe that there are only two fundamental types of antibody response: (1) to antigenic poisons (antitoxins) and (2) to foreign proteins (precipitins, agglutinins, complement fixing, etc.); in the latter case, the particular result elicited depends upon the particular set of conditions under which the serum is tested.

The chief characteristics of the specific serological reactions dealt with in this section may be briefly summarized as follows:

The agglutination reaction takes place when an antiserum is mixed with a specific test antigen which is always a suspension of discrete particles such as whole protozoa, bacteria, blood cells, etc. The reaction is evidenced by the clumping of the test antigen. As the clumps increase in size they generally precipitate.

The precipitation reaction takes place when an antiserum is mixed with (or layered under) a specific test antigen which to be satisfactory should be a clear solution. It is evidenced by the formation of a general precipitate when the two reagents are mixed or of a "ring" of precipitate when one is layered on the other.

The complement fixation reaction takes place when a solution of an antigen is mixed with its specific antiserum in the presence of complement (a thermolabile non-specific component of normal serums, guinea-pig serum, is generally used) but is not evidenced by any apparent change within the test tube. Consequently, after a sufficient incubation period, another antigen-antibody complex, *i.e.*, sensitized red blood cells, is added as an indicator. If the complement fixation reaction has taken place, the non-specific complement is fixed and cannot be utilized by the sensitized red cells, hence there will be no change in the test tube. On the contrary, if the reaction has not taken place, the non-specific complement is free, combines with the sensitized red cells and lysis takes place, thus liberating the hemoglobin of the red cells (see below).

Lysis causes the destruction of an organism by disintegration and is brought about by the action of at least two components of the antiserum: a thermostabile, specific component (amboceptor, sensitizer) and a thermolabile, non-specific component (complement, alexin). Furthermore, the parasite can be sensitized by *in vitro* contact with the amboceptor so that lysis occurs when complement is later added, whereas complement cannot combine either with the test antigen or amboceptor alone.

Phagocytosis also causes the destruction of an organism and consists in the engulfing of the organism by certain cells of the body whose action is greatly intensified in immunized hosts by opsonins which essentially sensitize the organism. Some immunologists (*cf.* Wells, 1925, p. 238), who look upon phagocytosis and lysis as being fundamentally the same, consider that a specific antibody sensitizes the invading parasite so that enzymatic lysis occurs, in one case outside the cell, in the other, inside. Levaditi and Mutermilch (1910) found that the *in vitro* phagocytosis of trypanosomes under the influence of immune serum possessed two phases: (1) the attachment of the trypanosome to the leucocyte, and (2) the actual process of incorporation of the parasite by the leucocyte. Although the second phase can take place only with live leucocytes, the first phase can take place between sensitized trypanosomes (parasites treated with immune serum) and leucocytes *in vitro* at 0° C., even if the leucocytes have been previously killed by a three-day sojourn in the ice-box, by heating to 45° 55° or 60° C., by successive freezing and thawing or by mechanical action. The sensitization of the parasites, *i.e.,* the action of the immune serum on the trypanosomes, falls essentially into the same category as the combination between lysin and specific organism.

Of these reactions, complement fixation is generally considered the most delicate, but in some cases agglutination is best. On the other hand, the precipitin reaction has the great advantage of simplicity, and can be used with far stronger test antigens than can complement fixation because the anticomplementary factor, to be discussed later, does not enter. In fact, in view of some recent work by C. I. Nelson, in which mutants of the same species of flax have been differentiated with the precipitin test, the superiority of the complement fixation test may be principally due to the concentrated attack that has been made on its standardization. This was a logical outcome of the success attending the non-specific Wassermann reaction in syphilis which utilizes similar technique.

SEROLOGICAL REACTIONS WITH EXTRACTS OF THE PROTOZOA: COMPLEMENT FIXATION AND THE PRECIPITIN TEST

A. Summary of methods of preparing test antigens. Inasmuch as the technical setting up of the tests has reached a fair degree of standardization, the experimental procedure at the outset of preliminary work pivots upon the satisfactory preparation of a test antigen. The attainment of this in agglutination tests is comparatively simple but in the complement fixation and precipitin tests depends upon a nicely balanced manipulation of conflicting, complex and often elusive factors, the control of which has taxed individual ingenuity unsparingly. At the outset the first step in the preparation of any test antigen depends upon the isolation of the parasite. This is fairly easy where the form is cultivable or free in the blood stream. Intracellular parasites, however, are not amenable to study by means of agglutination, phagocytosis and lysis but have been used in complement fixation and precipitation where extracts and not whole organisms are required. Even so, it is difficult to obtain concentrated solutions of the reactive parasitic material without including too high a percentage of extraneous host proteins. To obviate this, investigators have partially concentrated the parasites by centrifugation (*e.g.*, large schizonts of *Plasmodium falciparum* from minced infected placenta), or where the parasites are resistant to digestion have digested away the tissue, as in Bachman's work on coccidia.

Once the parasites are isolated, they are simply washed and resuspended in saline or distilled water for agglutination, phagocytosis or lysis. Sometimes they are killed for agglutination tests and it is theoretically possible to use dead organisms for phagocytosis. For complement fixation and precipitation they may be extracted, either fresh or after drying and pulverizing, in a variety of solvents. Alcohol and ether are common lipoidal solvents. Even when an aqueous solvent is eventually used, a preliminary extraction with lipoidal solvents often makes the later aqueous extraction more efficient. The aqueous solvents vary with almost every investigator. The simplest is distilled water or physiological saline. Solution of the protein often seems greatly facilitated by using a slightly alkaline solution, such as Coca's solution (an aqueous solution of 0.5% NaCl, 0.05% $NaHCO_3$, and 0.4% phenol); by stronger alkalies, such as N/10 NaOH or solutions of antiformin with subsequent neutralization with HCl before use; by 0.1% HCl which also has to be neutralized before use; or by various proportions of glycerin in saline. When necessary, various preservatives such as phenol, thymol, toluol may be added to inhibit bacterial action during extraction. In the actual test, the supernatant, either filtered or centrifuged or both, is used.

Although both lipoidal and aqueous solvents give rise to reactive test antigens, the reactive principles in each case may be different components of the original parasitic material. The great drawback to lipoidal solvents resides in the tendency of the resultant extract to react with syphilitic serums. This is not surprising in view of the remarkable reactivity of syphilitic serums in the Wassermann reaction where the test antigen is made up of a lipoidal extract of various *normal organs*. It may, however, be ruled out by dilution, *i.e.*, by ascertaining to what extent syphilitic serums react with a given test antigen and thereafter testing with higher dilutions of the test antigen.

B. Materials required. Since the complement fixation test is a delicate measure of whether or not complement is fixed, each of the ingredients has to be carefully titrated as far as possible. Furthermore, as the strength of the experimental antiserum and (until standardized) of the test antigen is unknown, it is extremely important to have a definite balance between complement, hemolytic antiserum and red blood cells, otherwise the test might be invalidated. For this reason, a standard has been arbitrarily accepted by most investigators and is as follows: The titre of the hemolytic antiserum is the smallest amount of undiluted, specific serum necessary to hemolyse completely one cubic centimeter of a five per cent suspension of blood corpuscles (one unit) in the presence of two units of complement. Two units of complement is generally about 0.08 of a cubic centimeter of fresh, normal, undiluted guinea-pig serum, but many investigators, following the earlier procedure, use 0.1 cubic centimeter. In some techniques, half, quarter or fifth units are frequently successfully employed for economy of materials. The latter are used in the following protocols which are patterned after the technique used by Prof. C. G. Bull of the Johns Hopkins University and are taken from a book by the present author (1929). Thus, 0.5 of a cubic centimeter of a two per cent suspension of corpuscles is one-fifth unit and 0.2 of a cubic centimeter of ten per cent guinea-pig serum (complement) is one-fifth of the older arbitrary dose of 0.1 of a cubic centimeter of undiluted serum.

In all cases control tubes are an essential accompaniment of every test to insure against one or another of the reagents, by themselves, causing a false reaction. Thus, in Table 1, Tube 17, by exhibiting no hemolysis, shows that the antiserum is not hemolytic by itself. Tubes 18 and 19 are likewise controls of the guinea-pig serum and saline, respectively.

Standard laboratory equipment, which is now available from laboratory supply houses, saves time and mistakes. Needless to say, it must be meticulously clean.

The necessary ingredients, besides the test antigen, are obtained as follows: The experimental, specific, inactivated (to destroy the activity of complement) antiserum is obtained from an animal infected or immunized with the experimental organism by bleeding aseptically, collecting the serum and heating for twenty minutes at 56° C. The complement is obtained by bleeding and collecting the serum aseptically from one or several healthy guinea-pigs. The unhemolysed, thoroughly washed red cells are obtained usually by bleeding a sheep aseptically into a bleeding bottle, containing such anticoagulents as heparin or sodium citrate, or glass beads for defibrination; and thereafter washing the cells three times in an excess of 0.85% saline (*i.e.*, mixing in saline, centrifuging at low speed, decanting the saline, adding more, etc.). The corresponding hemolytic antiserum is obtained by aseptically bleeding rabbits, which have been immunized by injections of sheep cells at various intervals, collecting the serum and preserving with half glycerin; in this state it will sometimes keep for a year or more. (Commercial, glycerinated antiserum of the needed high titre may now be bought.) It is to be noted that aseptic bleeding is an integral part of the procedure, proficiency in which is secured by practice. Blood may be obtained with a sterile hypodermic syringe from human beings by bleeding from the median basilic vein, from sheep from the jugular vein and from smaller animals from the heart; or if quantities of blood are needed from animals, by bleeding to death by cutting the jugular and collecting in sterile Petri dishes. (For more specific details, the reader is referred to Kolmer, 1928.) By preliminary titrations, the precise amounts of these ingredients are ascertained.

C. Protocols.[1] Protocols for the preliminary titrations are given in Tables 1 through 3. All amounts are given in cubic centimeters, each test tube having a total of two cubic centimeters.

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[1] The exact protocol used in each serological test varies with almost every laboratory. In the present review, I have selected only one procedure.

In order to facilitate discussion, hypothetical results are indicated.

Table I gives a protocol for the titration of the anti-sheep-cell rabbit serum which is the first reagent needing standardization. Inasmuch as this is an important basic titration, the complement should be standardized as far as possible (see following paragraph), and hence, should consist of pooled serum from several guinea-pigs. According to the possible results, complete hemolysis occurs through Tube 15 which contains 0.5 of a cubic centimeter of 1:10,000, and therefore, the titre is 1:20,000 in the fractional units or 1:4000 in the standard units. Later, in using sensitized cells, it is necessary to have cells and sensitizer in such a dilution that each 0.5 of a cubic centimeter contains two per cent cells and two units of sensitizer. This is easily done by mixing equal quantities of four per cent washed sheep cells and a dilution of sensitizer so that two units are contained in 0.25 of a cubic centimeter, which, in the example above, would be 1:2500, in other words, an equal mixture of double strength reagents. (A titration

TABLE I

PROTOCOL FOR THE TITRATION OF ANTI-SHEEP SENSITIZER

Tube	Sensitizer		Saline 0.85%	Complement (from 3 or more g.ps.) 1:10	Sheep cells 2%		Possible results
	Dilution	Amount					
		cc.	cc.	cc.	cc.		
1	1:100	0.8	0.5	0.2	0.5	Mix: Water bath at 37° C. 1 hour	Hemolysis
2		0.6	0.7	0.2	0.5		Hemolysis
3		0.4	0.9	0.2	0.5		Hemolysis
4		0.2	1.1	0.2	0.5		Hemolysis
5	1:1000	1.0	0.3	0.2	0.5		Hemolysis
6		0.8	0.5	0.2	0.5		Hemolysis
7		0.6	0.7	0.2	0.5		Hemolysis
8		0.5	0.8	0.2	0.5		Hemolysis
9		0.4	0.9	0.2	0.5		Hemolysis
10		0.3	1.0	0.2	0.5		Hemolysis
11		0.2	1.1	0.2	0.5		Hemolysis
12	1:10,000	1.0	0.3	0.2	0.5		Hemolysis
13		0.8	0.5	0.2	0.5		Hemolysis
14		0.6	0.7	0.2	0.5		Hemolysis
15		0.5	0.8	0.2	0.5		Hemolysis
16		0.4	0.9	0.2	0.5		No hemolysis
17	1:100	0.8	0.7	0.0	0.5	Controls	No hemolysis
18		0.0	1.3	0.2	0.5		No hemolysis
19		0.0	1.5	0.0	0.5		No hemolysis

for a given 1:10 dilution of the glycerinated antiserum will hold for about a week.)

Where the serums from a large number of healthy guinea-pigs are pooled, the unit of complement can be taken as a definite amount (in fifth units, 0.2 of a cubic centimeter of a 1:10 dilution). In practice, however, where complement is obtained from one or two animals, it is necessary to rule out individual variations by a preliminary titration. (See Table 2.) In the example given, since complete hemolysis occurs through Tube 4 the unit of complement is 0.1 of a cubic centimeter of a 1:10 dilution. In the actual fixation tests two units are used, or according to the titration in Table 2, 0.2 of a cubic centimeter of 1:10. A much lower dilution is not considered desirable. This titration has to be repeated for every batch of serum, or at least every other day when tests are run regularly.

TABLE 2

PROTOCOL FOR THE TITRATION OF COMPLEMENT

Tube	Complement 1:10	Saline	Sensitized cells	Possible results
	cc.	cc.	cc.	
1	0.30	1.20	0.5 Mix; incubate 1 hr.	Hemolysis
2	0.20	1.30	0.5	Hemolysis
3	0.15	1.35	0.5	Hemolysis
4	0.10	1.40	0.5	Hemolysis
5	0.05	1.45	0.5	None or partial
6	0.00	1.50	0.5 Control	None

The next step before performing the actual fixation test is to ascertain to what extent the test antigen will non-specifically fix complement. A protocol for this titration is given in Table 3, and in the example given, since partial hemolysis occurs in Tube 6, the anticomplementary dose is 0.6 of a cubic centimeter of a 1:20 dilution, and half of it can be considered a safe dilution for the actual test. Sometimes, it may happen that an experimental test antigen will be hemolytic, a condition which would be indicated if hemolysis occurred in Control Tube 10. In that event, the above protocol should be repeated leaving out the complement altogether. The subsequent dilution for the actual test would then have to be higher than the one that caused hemolysis of the cells and higher than the anticomplementary dilution. (Such titrations, once satisfactorily performed, will suffice for any particular batch of antigen.)

Having completed the preliminary titrations, the actual complement fixation test may be performed according to the protocol given in Table 4. In the example given, since no hemolysis occurs in Tubes 1 through 4, the complement was previously fixed by the experimental immune serum and test antigen (positive result), whereas had hemolysis occurred in all tubes, it could have been concluded that either immune serum or its specific antigen was absent (negative result).

As has been indicated previously, the Wassermann test for syphilis is carried out in the same manner as the specific complement fixation test, but it employs a *non-specific* test antigen which is generally derived from *normal* tissues by extracting with lipoidal solvents. Since normal organ extracts are used, it might be expected that positive Wassermann tests would frequently be found in other diseases. With modern methods of technique, however, such positives are not characteristic of other diseases, and a positive Wassermann test is quite presumptive of syphilis or the closely related yaws. Although protozoan diseases probably do not give positive Wassermann tests, it must be remembered that test antigens made from protozoa are extracts of cells and frequently adherent tissues, so that they often serve as efficient Wassermann antigens and give positive reactions with serums from cases of syphilis and yaws.

TABLE 3

PROTOCOL FOR THE ANTICOMPLEMENTARY TITRATION OF ANTIGEN

Tube	Antigen		Saline	Complement 1:10	Sensitized cells	Possible results
	Dilution	Amount				
		cc.	cc.	cc.	cc.	
1	undil.	0.3	1.0	0.2 Mix: Overnight in ice box or water bath for 1 hour.	0.5 Mix: Water bath 1 hour	No hemolysis
2	“	0.2	1.1	0.2	0.5	No hemolysis
3	“	0.1	1.2	0.2	0.5	No hemolysis
4	1:20	1.0	0.3	0.2	0.5	No hemolysis
5	“	0.8	0.5	0.2	0.5	No hemolysis
6	“	0.6	0.7	0.2	0.5	Partial
7	“	0.4	0.9	0.2	0.5	Hemolysis
8	“	0.2	1.1	0.2	0.5	Hemolysis
9	“	0.1	1.2	0.2	0.5	Hemolysis
10	undil.	0.3	1.2	0.0	0.5 Control	No hemolysis

The precipitin test is comparatively simple and easy to perform. As has been pointed out previously, the greatest obstacle is the preparation of a satisfactory test antigen. Besides the test antigen, antiserum, either from infected or immunized animals, is required. If enough reagents are available, a protocol similar to the one given in Table 6 for agglutination may be used, in which either the amount of test antigen is held constant and the amount of antiserum is varied, or the amount of antiserum is held constant and the amount of test antigen varied. If the reagents are at a premium, however, the "ring" test has proved efficient and

TABLE 4

PROTOCOL FOR THE COMPLEMENT FIXATION TEST

(The dilutions here are in accordance with the preliminary tests of Tables 2, 3, and 4)

Tube	Immune serum		Saline	Antigen 1:20	Comp. 1:10		Sensitized cells			Possible results
	Dilution	Amount								
		cc.	cc.	cc.	cc.		cc.			
1	1:4	1.0	0.0	0.3	0.2	Mix: Over night in ice box or water bath 1 hr.	0.5	Mix: water bath 1 hr.		No hemolysis
2	1:8	1.0	0.0	0.3	0.2		0.5			No hemolysis
3	1:16	1.0	0.0	0.3	0.2		0.5			No hemolysis
4	1:32	1.0	0.0	0.3	0.2		0.5			No hemolysis
5	1:64	1.0	0.0	0.3	0.2		0.5			Partial
6	1:128	1.0	0.0	0.3	0.2		0.5			Hemolysis
7	1:256	1.0	0.0	0.3	0.2		0.5			Hemolysis
8	1:512	1.0	0.0	0.3	0.2		0.5			Hemolysis
9	1:4	1.0	0.3	0.0	0.2		0.5		Controls	Hemolysis
10	1:8	1.0	0.3	0.0	0.2		0.5			Hemolysis
11		0.0	1.3	0.0	0.2		0.5			Hemolysis

TABLE 5

PROTOCOL FOR THE PRECIPITIN TEST ("RING" TEST)

Tube	Antiserum undiluted	Antigen *		Diluent	
		Dilution	Amount		
	cc.		cc.	cc.	
1	0.15	undiluted	0.15	0.00	
2	0.15	1:5	0.15	0.00	
3	0.15	"	0.00	0.15	Controls
4	0.00	undiluted	0.15	0.15	

* To be layered carefully on the antiserum.

consists in carefully layering about 0.1 of a cubic centimeter of the lighter fluid (generally the test antigen) onto a like amount of the heavier fluid (generally the antiserum) in small 5 by 50 millimeter tubes. A positive test shows a cloudy white precipitate of varying thickness, according to the potency of the reaction, at the interphase of the two liquids. The protocol in Table 5 has been used in various malarial and helminthological studies. (Further dilutions may be added if desired.)

SEROLOGICAL REACTIONS WITH SUSPENSIONS OF THE PROTOZOA: AGGLUTINATION, LYSIS AND PHAGOCYTOSIS

A. Preparation of antigens. The test antigen for these tests depends upon a homogeneous suspension (generally in saline, sometimes in distilled water) of thoroughly washed organisms, either dead or alive. The need of a carefully standardized suspension of organisms, especially in the agglutination test, is apparent when it is realized that any preliminary clumping or aggregation will invalidate the end result.

TABLE 6

PROTOCOL FOR AGGLUTINATION TEST

Tubes	Antiserum			Suspension of Organism	Diluent
	Optional Dilutions		Amounts		
			cc.	cc.	cc.
1	1:10	1:5	0.5	0.5	0.0
2	1:20	1:10	0.5	0.5	0.0
3	1:40	1:20	0.5	0.5	0.0
4	1:80	1:40	0.5	0.5	0.0
5	1:100	1:80	0.5	0.5	0.0
6	1:120	1:160	0.5	0.5	0.0
7	1:140	1:320	0.5	0.5	0.0
8	1:160	1:640	0.5	0.5	0.0
9	1:180	1:1280	0.5	0.5	0.0
10			0.0	0.5	0.5 Control

Mix and incubate 1 hr. at 37° C.; refrigerate 12 hrs.; examine the tubes macroscopically or with a hand lens.

B. Materials required. The agglutination reaction has been used to ascertain (1) whether an animal is infected with a certain organism or (2) whether an organism under examination is identical with one used in obtaining a particular agglutination serum. In the first case, varying dilutions of serum from the experimental animal are mixed with the known organism; in the second case, the experimental organism is mixed with an agglutinating serum produced with a known organism. The materials required are the specific test antigen and antiserum. As various substances, particularly saline, sometimes cause agglutination by themselves, the control tubes are very necessary.

C. Protocols. The protocol in Table 6 which is set up in 8 by 100 millimeter tubes has been used extensively. Dilution, which may be extended indefinitely, should be carried out far enough to rule out natural and group agglutinins. Whether such agglutinins exist should be ascertained by control tests with normal serum and serum from animals either infected or immunized with related organisms.

The microscopic method for the agglutination reaction has been developed where only a small amount of blood serum or dry blood is obtainable. It is most widely used for the Gruber-Widal reaction for typhoid fever and may be outlined as follows:

Cover-slip	*Blood Serum*		*Homogeneous Moderate Suspension of Organisms*	*Diluent*
	Dilution	Amount		
1	1:20	Platinum loopful	Platinum loopful	
2	1:40	" "	" "	
3	Control	0		Platinum loopful

Mix and invert each cover slip over a vaselined hanging-drop (concave) slide. Place in the dark. At the end of one hour examine with a microscope for clumping and motility, being sure that the control slide shows none. With dried blood the procedure is essentially the same except that the blood can only be approximately diluted.

In vitro lytic experiments with the protozoa can be carried out in the same manner as hemolysis, as given in Table 1. Since physiological saline frequently causes disintegration of protozoa (especially among the pathogenic trypanosomes) upon incubation, Control Tube 19 is impossible. Dilutions can of course be varied to suit individual requirements.

In vitro phagocytosis experiments, according to Levaditi and Mutermilch (1910), may be carried out by mixing equal quantities of immune serum and blood containing parasites and leucocytes, and, subsequently, studying microscopically for evidence of phagocytosis. In such work, controls have to be used to ascertain how much the unsensitized organisms (without immune serum) are phagocyted by the leucocytes.

MISCELLANEOUS SEROLOGICAL TESTS

A great deal of interest has been aroused in the past few years by certain tests in kala-azar. Thus, Brahmachari (1917) noted that the addition of distilled water to serum from kala-azar cases resulted in the formation of a copious precipitate which was globulin in nature and from which he devised his so-called serum globulin test for the disease. Ray (1921), in using a hemoglobinometer, noted that with blood from kala-azar patients a cloudiness developed, which he thought was due to incomplete hemolysis of the red cells (hence his name, "hemolytic test"), but which later work showed came from the serum and was also globulin. (See Sia and Wu's, 1921, work on the globulin precipitation test.) Hill (1913) had probably observed the same phenomenon in making leucocyte counts. Gaté and Papacostas (1920) devised what they termed the "formol gel test" for syphilis; this consisted in adding two drops of commercial formalin to one cubic centimeter of clear serum, whereupon syphilitic serums formed a hard gel within twenty-four to thirty hours and normal serums did not. In applying this test to kala-azar, Spackman (1921) found that the serum not only jelled within a few seconds, *but became opaque.* This opacity is described by Napier (1922) as resembling coagulated white of egg.[2] Still another flocculation test, *i.e.,* the antimony test, was suggested by Chopra, Gupta and David (1927), who found that a copious precipitate was formed when kala-azar serum was brought in contact with antimony derivatives, especially urea compounds. Furthermore, Napier (1927) found that the efficacy of a number of pentavalent antimony compounds varied directly with their therapeutic efficiency. All of the tests seem to be dependent upon an increase of euglobulin in the serum. (See Brahmachari, 1917 and 1923; Napier, 1922; Ray, 1924 and 1927.)

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[2] The opacity needs to be stressed. Since the original test, which was dependent on jellification alone, was found to be unreliable, some investigators have likewise condemned the test in kala-azar. In doing this, they overlook the fact that, although the procedures are similar in the two tests, the criteria are different. To obviate this confusion, Napier has suggested calling the kala-azar test the aldehyde test. Using his criteria, the test has proved to be very specific for kala-azar, except for complications with schistosomiasis. (*Cf.* Faust and Meleney, 1924.)

The tests are extremely simple to perform. Thus, in Brahmachari and Sen's (1923) modification of Brahmachari's test, serum is mixed with six times its volume of distilled water and poured into a graduated cylinder until some black spots (previously fixed at the bottom) are just visible. The height in inches varies inversely with the amount of precipitate and a reading of 1.25 inches or less is regarded as positive. In Napier's aldehyde test, one drop of commercial formalin (thirty per cent formaldehyde) is added to one cubic centimeter of serum; the mixture is shaken and put at room temperature. Kala-azar serum will set and become opaque in from three minutes to twenty-four hours.

SEROLOGICAL INVESTIGATIONS AMONG THE PROTOZOA: RESULTS AND SPECIAL TECHNIQUES

A. Amœbiasis. The serology of amœbiasis will not be considered because it is taken up in detail by Dr. C. F. Craig in another chapter of this book.

B. Leishmaniasis. Most of the work on leishmaniasis has been done with kala-azar and available data indicate that in this disease antibodies are difficult to demonstrate, appear very variably, and, so far, offer little promise of perfecting standardized diagnostic tests. Since killed cultures of *L. donovani* stimulate the production of antibodies of high titre when injected into man or animals, since the serums from recovered cases of the disease apparently manifest high titres and since kala-azar is essentially an infection of the reticulo-endothelial system which is probably most active in the production of antibodies, the lack of high titre in this disease may be due to a blockade of the reticulo-endothelial system by the parasites. In complement fixation, many investigators have used extracts of infected spleens (see especially di Cristina and Caronia, 1913, and Hindle, Hou and Patton, 1926); others have used cultural flagellates (Auricchio, 1927). The agglutination studies have been done with suspensions of cultural flagellates.

In marked contrast to the specific serological antibodies, very promising results have been obtained in kala-azar with the miscellaneous tests. Tables 7 and 8 are representative of the many papers on these tests. The data given by Napier (1923) in Table 7 show the correspondence between the aldehyde test and the finding of parasites by various methods. The comparative tests by Chopra, Gupta and Basu (1927) in Table 8 affirm the superiority of the antimony test over the aldehyde test. As may be seen from the latter, the tests are not dependable for very early diagnosis and do not as yet seem to supplant the demonstration of the parasite to establish the disease.

TABLE 7

CORRELATION OF ALDEHYDE TEST FOR KALA-AZAR AND RESULTS OF SPLEEN PUNCTURE

(Data obtained by combining tables given by Napier, 1923)

Aldehyde test

Spleen examination		+	?	—	Totals
	+	191	32	8	231
	—	10	16	96	122*
	Totals	201	48	104	353

* Of these 122 patients negative by spleen puncture, 14 were hospital patients and had the following examinations: 1 had a single spleen puncture, 8 had two, 4 had three and 1 had four.

C. Trypanosomiasis. Although the diagnosis of trypanosomes rests in general on the demonstration of the specific parasite, the scarcity of parasites in some infections makes serological studies of interest, and in one case (dourine of horses) serological tests have been found to be superior to routine microscopical examination and are used extensively in certain laboratories.

TABLE 8

A COMPARISON OF THE ANTIMONY AND ALDEHYDE TESTS WITH SERUMS FROM PATIENTS INFECTED FOR VARYING LENGTHS OF TIME WITH KALA-AZAR

(From Chopra, Gupta and Basu, 1927)

	Antimony test			Aldehyde test		
Kala-azar cases	+	?	—	+	?	—
Duration:						
10–12 days	0	3	0	0	0	3
15–21 days	12	0	0	0	0	12
1–3 months	30	0	0	0	4	26
Various lengths	235	21	0	184	40	32
Other diseases	18	26	181	10	39	176

In an extended series of investigations on complement fixation the test antigens have been of three types: (1) extracts of normal tissues, thus amounting to a non-specific Wassermann test; (2) extracts of organs from animals infected with trypanosomes; and (3) extracts of parasites which have been more or less completely isolated from the blood and body fluids of the host.

Work on the non-specific Wassermann test was initiated by Landsteiner, Müller and Pötzl (1907) and taken as a whole gives rather inconsistent results. The following conclusions are, however, probably justified: Serums from rabbits infected with various species of trypanosomes frequently give positive Wassermann reactions. In this connection Landsteiner and van der Scheer (1927) have shown that the serums of rabbits become strongly Wassermann positive when the animals are immunized with dead trypanosomes (*T. equiperdum*), but less regularly so when they are infected. (Since serums from seemingly normal rabbits also frequently yield positive Wassermann reactions, most investigators have been careful to use only animals which are negative before they begin their experiments.) Serums from other infected animals sometimes show positive Wassermann tests, but are too inconstant to be used for diagnosis. Serums from man infected with trypanosomiasis uncomplicated by syphilis or yaws, in spite of several positive reports, probably do not give positive Wassermann tests, especially with the modern perfection of technique.

Della Vida (1907) and Weber (1907) were probably the first to attempt specific complement fixation in trypanosomiasis and used extracts of organs from infected animals. With the exception of Hartoch and Yakimoff (1908) the early work was in the main inconclusive. This, Levaditi and Mutermilch (1909) attributed to the use of weak non-purified test antigens. Accordingly, they isolated their trypanosomes from blood constituents by washing and centrifuging; then they either suspended them in saline or after drying *in vacuo* over sulphuric acid and pulverizing, extracted 0.05 gram of the powder in four or five cubic centimeters of saline at 37° C. for one hour. Working with *T. evansi* and several strains of *T. brucei*, they obtained such specific results for the genus (not for the species) that they concluded the test could be used to detect infection, but not specific infection. McIntosh (1910), in parallel Wassermann and specific complement fixation tests, concluded that the latter gave stronger reactions.

Subsequently, rapid strides were made during the course of some very careful work from which evolved the standardization of the complement fixation test for diagnosing dourine of horses, caused by *T. equiperdum*. Some of the men associated with this work are Winkler and Wyschelessky (1911), Mohler, Eichhorn and Buck (1913), Watson (1914, 1915, 1920),Wehrbein (1914), Waldman and Knuth (1920), Dahmen (1922), Bessemans (1921, 1922) and Bessemans and Leynen (1922, 1923). Of especial interest are the following details: As test antigen Mohler, Eichhorn and Buck used freshly prepared extracts of spleens of rats heavily infected with *T. evansi* or of trypanosomes isolated by laking the blood cells with saponin. Watson (1920) decided that the test was specific in diagnosing the disease and was often positive before symptoms were apparent or during latent stages. As test antigen, he used trypanosomes isolated by washing and centrifuging first in citrated saline and later in saline, preserved in twice their volume of an extracting fluid (0.85 per cent NaCl, 90 parts; pure neutral glycerin, 10 parts; and formol, 1 part) and kept on ice in one cubic centimeter ampules. Reynolds and Schoening (1918) modified this technique by laking the blood cells in distilled water. Dahmen, in testing the suitability of alcoholic and carbolized saline extracts, concluded that the active components were different in each and that the alcoholic was more potent. In making the latter, the concentrated trypanosomes were dried at 50° C. and pulverized; 0.1 gm. of powder was shaken an hour in one cubic centimeter of ether; the residue, after filtration, was allowed to stand in one cubic centimeter of absolute alcohol two days at 37° C.; and finally, the supernatant was slowly diluted with five to ten volumes of water. Bessemans (1922*b*), in a very detailed study of the anticomplementary and antigenic power of organ and isolated trypanosome test antigens extracted in alcohol and ether, found that emulsions of trypanosomes isolated by centrifugation and diluted in the alcoholic or aqueous extracts of Mohler or Watson were most efficient.

Some serological method is especially needed in Chagas' disease where definite diagnosis can only be made by finding trypanosomes in the blood during the short acute stage of the disease or at autopsy by finding the leishmanial forms in the tissues. Very promising results were reported by Machado and Guerreiro (1913), Villela and Bicalho (1923) and Lacorte (1927). In all of this work, however, it is difficult to evaluate the correspondence between the serological tests and the presence of infection because criteria for the latter are so uncertain.

Just as in the complement fixation studies in trypanosomiasis, the most extensive work on precipitins has been concerned with attempts to devise a diagnostic test for dourine. Precipitins were probably first demonstrated in trypanosomiasis by Mayer (1905) with saline extracts of *T. brucei* after digestion for a week with trypsin. Uhlenhuth and Woithe (1908) obtained positive results in dogs and horses infected with *T. equiperdum* but negative ones in infected rabbits. The first successful work with dourine infections, which compared favorably with complement fixation tests, was reported by Winkler and Wyschelessky (1911) and was confirmed by Ruppert (1912) with test antigens prepared by extracting isolated trypanosomes in from ten to twenty parts saline in a shaking machine for from one to three days, centrifuging and filtering. They used the ring test. Lanfranchi (1912) reversed the usual procedure (tested for antigen instead of antibody) by mixing serums of animals infected with *T. brucei* or *T. equiperdum* with serums of dogs highly immunized with *T. brucei;* although he reported favorable results, the experimental details are too few to evaluate the method.

Dahmen (1922) concluded that alcoholic extracts were superior to aqueous in the precipitin test and with them he devised his so-called lipoid precipitation method which he considered superior to complement fixation. Fuest (1922) also concluded that Dahmen's lipoid precipitation method with alcoholic extracts was more sensitive than complement fixation, as manifested by these facts of 1016 horses studied, 83 reacted positively in the precipitin test, 73 in complement fixation; of 56 known-infected horses, 53 reacted positively in the precipitin test, 40 in complement fixation.

Agglutination of *T. lewisi* by immune rat serum was noted in 1900 by Laveran and Mesnil and has been repeatedly observed in this and other species by many observers. Among the pathogenic trypanosomes, prior to 1911, only low titres were obtained with little indication of more promising results, but thereafter it was clearly demonstrated that with proper technique, agglutinins of high titre could be demonstrated in animals either infected or immunized. Lange (1911) found that normal serums never agglutinated trypanosomes in dilutions higher than 1:100, but that serum from animals immunized with dead trypanosomes showed titres as high as 1:1600 and from infected animals might reach 1:12,800. Group reactions (though to a less extent) were obtained with *T. equiperdum*, *T. brucei* and *T. gambiense*. The test antigens consisted of washed trypanosomes suspended in saline and preserved in formalin. This work was corroborated and amplified to include other trypanosomes by Winkler and Wyschelessky (1911) who indicated that the test might be of diagnostic value in dourine and compared favorably with parallel complement fixation and precipitin tests; by Mattes (1912); by Ruppert (1912); by Lanfranchi (1912) who obtained signally high titres by a technique not described; by Offermann (1915) who concluded from a study of rabbits during the course of infection with *T. equiperdum* that complement fixation was somewhat superior to agglutination; and by Marcone and de Gaspari (1921).

A long series of investigations are in accord in demonstrating that the serum of many animals develops a trypanolytic property which is associated with periodic disappearances of the organisms from the blood. Rodet and Vallet (1906) conducted a valuable study of the lysins arising during the course of uninfluenced infections of the pathogenic trypanosomes. This work was continued and amplified by Massaglia (1907), Levaditi and Mutermilch (1909), Leger and Ringenbach (1911 and 1912) and Mutermilch and Salamon (1928).

D. Malaria. Malaria has attracted a great deal of attention because various observers have reported that different stages of the disease, especially of æstivo-autumnal, yield positive Wassermann tests and a few have gone so far as to suggest its use in the diagnosis of malaria. Nevertheless, at present, the general trend of opinion seems to be that malaria, without concomitant yaws or syphilis, only yields positive Wassermann tests with faulty technique. Among several recent excellent reviews of the subject attention may be called to that of Lloyd and Mitra (1926) who pointed out that: (1) the so-called Wassermann test has been carried out in a variety of ways; (2) the highest proportion of positive tests in malaria has been obtained by workers using Wassermann's original method; (3) in many positive tests syphilis has been inadequately excluded; and (4) workers using modern techniques have almost invariably found negative tests in malaria.

The chief difficulty in the study of specific complement fixation and precipitation in malaria lies in isolating an intracellular parasite and obtaining a sufficiently high concentration of parasitic materials in the test antigens. Passing over a series of negative or very conflicting results, Gasbarrini (1913 and 1914) reported quite promising results with a test antigen prepared by washing heavily parasitized red cells in 0.9% saline, laking in distilled water, drying in a desiccator, pulverizing, and for use, diluting 1:30 with saline. He obtained positive results only when he absorbed the human serum with sheep cells to remove the natural antisheep antibody.

Thomson (1918 and 1919) also obtained promising results with an antigen prepared from parasitized cells, first cultivated by a modification of Bass and Johns' (1913) technique; then after hemolysing the red cells, the sedimented parasites and cell débris were dissolved in N/10 NaOH, neutralized with normal HCl and just before use diluted with physiological saline until there was no anticomplementary action. Preliminary results with a heavily infected spleen appeared worthy of further investigation. A group reaction occurred among the various malarial parasites and pseudo-positives with syphilitic serums.

Horowitz-Wlassowa (1924) obtained parasites from localizations in the placenta and brain, extracted them for twenty-four hours in a 0.1% solution of quinine hydrochloride containing a few crystals of thymol and filtered. He concluded that the complement-fixing antibody is formed in malaria but depends upon varying conditions and may be present from two weeks to five years after infection, although during reinfection or relapse, when the parasites, and especially the gametocytes, are increasing in the blood, it is not demonstrable. Savtchenko and Baronoff (1926) found infected livers more efficient than spleen and, unlike other workers, obtained definite species specificity. Kingsbury (1927) felt that saline emulsions of washed and hemolysed infected cells were superior to tissue extracts. Manson-Bahr (1927) prepared alcoholic extracts of oöcysts of plasmodia from the stomachs of mosquitoes, and although he failed to obtain evidence of complement fixation, he believed that if the test antigen could be made stronger it might prove satisfactory.

Pewny (1918) first obtained precipitins in malaria with a test antigen prepared from heavily parasitized blood clots (placenta?). These were digested in distilled water at 37° C. for 5-6 days, centrifuged and filtered. The present author has been associated with two studies of precipitins in malaria (W. H. and L. G. Taliaferro, and Fisher, 1927, and W. H. and L. G. Taliaferro, 1928). The most efficient test antigen was obtained by concentrating the parasites from a heavily infected placenta by centrifugation; these, either dried or fresh, were extracted in ether; and the wet residue was extracted in Coca's solution, filtered and adjusted to pH 7.8 before use. Some work was also carried out by extracting 0.05 gm. dried powder for from ten to twenty hours in from one and one-half to two cubic centimeters N/20 HCl made up in saline, and adjusting the supernatant to pH 7.8 before use.

E. Coccidiosis. Although a number of the earlier workers felt that coccidiosis in the rabbit incited the formation of Wassermann reagins and might thus explain why so many "normal" rabbits were Wassermann positive, more recent work indicates that with carefully controlled technique coccidiosis of the rabbit does not lead to positive Wassermann tests (Marcuse, 1922).

Specific complement fixation was considered of no diagnostic value by Marcuse (1922) but of considerable value by Patterson (1923) who found normal saline extracts of infected livers to be best. Chapman (1929) did not obtain very high titres although she felt that the test was probably specific. The most satisfactory test antigen was prepared by shaking a heavily infected minced rabbit-intestine in fifty cubic centimeters of distilled water plus 0.5% phenol for two hours, leaving at room temperature two days, adjusting to isotonicity with NaCl, centrifuging and filtering.

It is impossible to decide whether these variable results are due to lack of an efficient antigen or to lack of antibody formation. Prof. G. W. Bachman (unpublished work) has succeeded in preparing a powder of pure *Eimeria* from rabbits by digesting away the muscle fibers in pepsin and hydrochloric acid and getting rid of remaining yeasts by a differential flotation with a sugar solution, then washing and drying. He has found only weak precipitin reactions, but the benzoin floculation test of de la Rivière (1929) gives considerable promise.

SEROLOGICAL TESTS USED IN CLASSIFICATION

In the foregoing sections, the specific nature of the various serological reactions has been emphasized. This specificity is not absolute, however. It generally shows quantitative differences closely paralleling zoölogical and botanical classifications (which are based on anatomical criteria) so that in general the more closely two species are related the stronger the group reaction between them. Consequently, serological tests have been used for many years to check and amplify other biological classifications. Since much higher titres as a rule may be attained with immunized than with infected animals, most of this work, and especially the more recent work, has been carried out with immunized animals, and in particular with immunized rabbits.

Cross serological tests have been used among the *Leishmania* infections (*L. donovani* of kala-azar, the similar infections of dogs, *L. infantum* of infantile kala-azar,[3] *L. tropica* of oriental sore and *L. brasiliensis* of American leishmaniasis), because they cannot be differentiated morphologically. They have also been used in studying the relationship of the *Leishmania* to various insect and plant herpetomonads which bear a great resemblance to the cultural forms.

Some early work by Bandi (1913) with the agglutination test and by di Cristina and Caronia (1913) with the agglutination and complement fixation tests indicated the identity of *L. infantum* and the parasite of the canine infection. Noguchi (1924) distinguished three varieties of *Leishmania* (*L. donovani, L. tropica* and *L. brasiliensis*) by cross agglutination tests. He obtained titres of 1:10 and 1:100 with immunized rabbits and living cultures. Kligler (1925) confirmed this work in the main. Using the same test but with standardized test antigens of killed organisms Wagener and Koch (1926) found differences not only between the three varieties just mentioned but also between them and *L. infantum*. Moreover, none showed any relationship to *Herpetomonas ctenocephali*. They obtained titres with homologous antiserums of 1:80 up to 1:600. In the same year, Noguchi (1926), by means of agglutination and complement fixation reactions, obtained no indication of a relationship between the various *Leish-*

[3] The three visceral infections, *i.e.*, kala-azar, infantile kala-azar and the canine disease, are probably caused by the one parasite, *L. donovani.*

mania strains and *Herpetomonas* strains from plants and insects. In view of Noguchi's former conclusion that *L. donovani* and *L. infantum* were identical, it is worthy of note that in the present work, the two showed marked differences.

An interesting use of serological tests was made by Adler and Theodor (1926) who tried to identify by cross agglutination tests, a protozoön isolated from a vertebrate host, with stages found in invertebrate hosts captured in nature. According to them *L. tropica* and *H. papatasii* are identical.

There has been comparatively little work done on the serological reactions among the trypanosomes, mainly because interest was absorbed in other types of tests (*i.e.,* cross immunity tests). Moreover, at first, they were considered to be only genus-specific, but their efficiency might well be reexamined in light of the recent tendency of authorities, like Wenyon (1926), to combine so many of the formerly recognized species. For example, Mattes (1912), using agglutination tests with *T. congolense, T. brucei, T. equiperdum* and *T. gambiense,* found differences in titre between homologous, as compared to heterologous, antiserums and test antigens. Levaditi and Mutermilch (1910*b*), using the phenomenon of "attachment" (initial stage of phagocytosis), differentiated *T. brucei, T. togolense* (a strain of *T. brucei*), *T. dimorphon* (partly *T. congolense*) and *T. gambiense.* Others used *in vitro* trypanolysis (see previous citations in section on *Trypanosomiasis*). Robinson (1926), using complement fixation tests, found that the serums of animals infected with *T. congolense* reacted with the homologous test antigen, but not with *T. equiperdum,* whereas a group reaction existed between *T. brucei* and *T. equiperdum.*

The serological reactions considered in this chapter are not the only ones that have been used in classification. Cross immunity after recovery from infection with the trypanosomes, leishmanias and piroplasms has been extensively used. Cross immunity after cure with drugs has been used to differentiate strains of trypanosomes, but is too delicate to be of much use in classification. Thus, by incompletely curing a mouse of trypanosomiasis twenty times, Ritz (1914) obtained seventeen immunologically different relapse strains which could be sharply differentiated by cross immunity tests. It is obvious that so delicate a test could not be used as a criterion for species. This, however, brings up a seeming contradiction which is of tremendous interest immunologically. Many of these immunity tests indicate a greater antigenic difference between the relapse variants of one strain of trypanosomes than between what are generally recognized species of trypanosomes. For example, Braun and Teichmann (1912) found that mice immunized with *T. equiperdum* showed a group immunity, *i.e.*, were immune to *T. brucei* and *T. evansi,* although they were not immune to a serum resistant (relapse) strain of *T. equiperdum.* The same situation is evident in many of the serological tests. Thus, a given trypanolysin may be reactive not only against the original strain which stimulated its production but against a number of related species (Leger and Ringenbach, 1912), whereas it may not be reactive against the trypanosomes (relapse strain) reappearing in the body of the same animal in which the trypanolysin was formed.

FUNCTIONAL RÔLE OF ANTIBODIES IN MODIFYING THE COURSE OF INFECTION AMONG THE PROTOZOA; LYSIS, PHAGOCYTOSIS AND INHIBITION OF REPRODUCTION

The reactions, which have been considered *in vitro,* can undoubtedly take place within the body under suitable conditions. Thus, complement fixation is simply an expression of the fact that complement may combine with a specific antibody and a specific antigen or test antigen. Agglutination has been shown by the work of Bull and others (see review by Bailey, 1928) to be probably of functional importance in the natural and acquired immunity of animals to bacteria. Little has been done on this phase of the subject in protozoölogy. There are other reactions, however, *viz.*, lysis, phagocytosis and inhibition of reproduction, which have a direct effect on the parasites and which can be shown to alter the course of infections in animals. It should be emphasized that the consideration of complement fixation, agglutination, precipitation, lysis, phagocytosis and inhibition of reproduction, as such, does not imply that there are so many *different* antibodies. Many immunologists believe that the first five are manifestations of the same antibody tested under diverse conditions, while the last mentioned, according to available evidence, is probably due to a separate one.

The protozoa are preeminently fitted, because of their comparatively large size, for a study of the *in vivo* action of antibodies and their functional rôle in influencing the course of infections. Particularly is this true of certain trypanosome and malarial infections where the entire life cycle occurs in the blood stream and representative samples can be obtained from the peripheral blood.

By making daily number counts of such blood protozoa and studying the resulting number curves, various types of infections can be differentiated in experimental trypanosome and malarial infections. The simplest condition is seen when the parasite reproduces at a constant uninterrupted rate and the organisms accumulate in the blood stream according to a geometrical progression series until the host dies. Such an uninterrupted increase of the organisms can be modified by one or both of two antibodies, *i.e.,* the parasites may be destroyed by means of lysis or phagocytosis after they are formed, or reproduction (cell division) may be inhibited by means of a reproduction-inhibiting antibody.

The reproduction-inhibiting property of serum (W. H. Taliaferro, 1924) inhibits the reproduction of the parasites and is brought about by the action of immune serum. As it has only been carried out in *in vivo* experiments, whether or not complement is necessary has not been ascertained. It differs from lysis in that there is no affinity between antigen and antibody *in vitro* (*i.e.,* the antibody does not sensitize the antigen).

A. Trypanosomiasis and avian malaria. The effect of these various processes may best be illustrated by specific examples which are taken from reviews by the author (1926, 1928, and 1928*a*). The simplest type of infection is encountered when the pathogenic trypanosomes are grown in the mouse where no demonstrable antibody whatever is developed, as evidenced by the constant reproductive rate and the steady accumulation in numbers of parasites.

When the same trypanosomes are grown in the guinea-pig, rat, rabbit, dog, etc. (W. H. and L. G. Taliaferro, 1922) or when the bird malarial parasite is grown in the canary (L. G. Taliaferro, 1925), a parasiticidal resistance is formed as evidenced by the unchanging rate of reproduction throughout the infection in conjunction with the irregular but periodic number crises. In the trypanosome infections the parasiticidal resistance has been shown to be a lysin (*in vitro* by Schilling, 1902; Lingard, 1904; Franke, 1905; Rodet and Vallet, 1906; Massaglia, 1911 and 1912; and *in vivo* by Diesing, 1905; Kleine and Möllers, 1906; the author and Johnson, 1926; and Johnson, 1929). In the avian malarial infection it has been shown not to be a humoral antibody (W. H. and L. G. Taliaferro, 1929), but is associated with a heightened activity of the reticulo-endothelial system (Cannon and W. H. Taliaferro, unpublished).

When the non-pathogenic *T. lewisi* is grown in the rat (W. H. and L. G. Taliaferro, 1922; Coventry, 1925; and Regendanz and Kikuth, 1927), and guinea-pig (Coventry, 1929), both types of antibodies are formed as manifested by the retardation and final inhibition of the rate of reproduction about the tenth day in both animals, coupled with the two definite number crises (one at approximately the tenth day and the other at the termination of the infection in the rat) and the scarcity of parasites throughout an infection in the guinea-pig. The basis for the inhibition of the rate of reproduction (as studied by W. H. Taliaferro, 1924 and Coventry, 1925), has been found to be due to the acquisition of an immune property, by the serum of infected rats, which inhibits cell division but which does not kill the parasites. The first number crisis on about the tenth day in the rat has been found to be due to a passively transferable trypanocidal property, probably a lysin (Coventry, 1929) as has also the second number crisis which terminates the infection (MacNeal, 1904; Manteufel, 1909; W. H. Taliaferro, 1924; Coventry, 1929), with phagocytosis possibly playing a subsidiary rôle (Laveran and Mesnil, 1901; and Regendanz and Kikuth, 1927).

The methods used in this work consist in ascertaining: (1) what type of number curve a given infection shows, and (2) whether the reproductive activity remains constant or changes. Thus, to cite one possibility, if the reproductive activity remains constant while the number of parasites decreases (or even remains at a level), a parasiticidal mechanism is operating.

The number curve is easily found by getting the proportion of parasites and red blood cells per cubic centimeter at frequent intervals.

Among the trypanosome infections, the reproductive activity is obtained indirectly by ascertaining the coefficient of variation for total length of samples of parasites from daily blood smears throughout the infection, because if reproduction is rapid, there will be a great variability in size, but if it does not occur, there will be little variability. The coefficient of variation is ascertained in the following way:

(1) From each blood smear 50 or 100 parasites are found at random and are drawn by means of a camera lucida at a convenient magnification.
(2) The total length of each parasite is found.
(3) The mean $\left(\frac{\Sigma x}{n}\right)$ of each sample of 50 or 100 total lengths is obtained. (Σx = the sum of the lengths; n = the number drawn.)
(4) Then the standard deviation $\left(\sqrt{\frac{\Sigma x^2}{n} - M^2}\right)$ is obtained. (Σx^2 = the sum of the squares of the lengths; n = the number drawn; M^2 = the square of the mean.)
(5) Finally, the coefficient of variation $\left(\frac{\sigma 100}{M}\right)$ is obtained. (σ = the standard deviation, M = the mean.) If the coefficient of variation remains constant, the rate of reproduction may be said to remain constant, etc.

Since the malarial parasites show a synchronicity in their reproductive activity, their rate of reproduction can be obtained directly by ascertaining whether the cycle of growth and sporulation takes the same length of time throughout an infection. The methods for ascertaining the cycle of growth and sporulation as well as its bearing on the rate of reproduction may be found in Chapter XXXVIII by L. G. Taliaferro.

The work just reviewed leads directly to a consideration of various aspects of the functional rôle of antibodies, such as the curative and protective action of immune serums, the artificial production of immunity in animals and the cellular basis of antibody production and immunity to protozoa in general. For these phases of the subject, the reader is referred to W. H. Taliaferro (1929).

CHAPTER XLI

STATISTICAL METHODS IN PROTOZOÖLOGY

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The statistical method has made its entry into the various fields of biology through a desire on the part of the workers in these fields to add a quantitative element to their qualitative descriptions. This has usually resulted in a few early experiments and papers giving a simple set of measurements on some particular characteristic, usually the size of a biological organism. At the start, such measurements have been given individually and it is only after the accumulation of a series of such measurements that a real need for the statistical method is felt. The introduction of the statistical method into any particular field for the purpose of simple description of size is quickly followed by its use for the purpose of describing more complex characteristics, such as biological variability, relationship between variables and rates of change with time. The field of protozoölogy has, in recent years, been following the natural course of evolution with regard to the statistical method, and it is the purpose of this article to present certain elementary statistical procedures and to point out their applicability in the field of protozoölogy.

STATISTICAL DESCRIPTION OF MEASUREMENTS ON A SINGLE VARIABLE

When measurements are taken on a single variable and they are few in number, it is a perfectly satisfactory logical process to record these measurements and treat them individually. When, however, we have taken a large number of such measurements, we find that this process cannot be followed, for the mind fails to get a clear view of the entire series. For this reason, the statistical treatment starts by arranging these measurements in order, according to size.

For example, if we were measuring the length in microns of trophozoites of *Giardia bradypi,* as was done by Hegner and Schumaker (1928), and we had taken measurements on 100 of these forms, we would find it necessary to arrange these measurements in what is called a frequency table. Such an arrangement is illustrated in Table I, which gives the frequency distribution of measurements found by these experimenters.

TABLE I.

Length in Microns	Frequency
10	1
11	6
12	25
13	32
14	32
15	3
16	1
Total	100

Quantitative information of this sort is presented graphically as in Fig. 29. Bars are used to indicate the frequency because the measurements, although recorded as 10, 11, 12, etc., microns, are in fact distributed at various points about these exact positions and the set of measurements recorded at any particular position such as 12 represents measurements of values that occurred between 11.5 and 12.5.[1]

Such a table with its accompanying graph presents the facts with regard to these measurements in a form that gives us considerable information as to the length of these organisms. We see that they tend to center in size somewhere in the neighborhood of thirteen to fourteen microns and that the smallest organism observed was ten microns in length and the largest one, sixteen. This simple presentation of the material involves two of the ideas that form the basis of the simple description of any frequency distribution; they are, namely—the concept of measurements centering at some point and the concept of deviation or variation of such measurements from this centering point. Other ideas than these are used in the field of statistics in describing frequency distribution, but these two are fundamental.

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[1] The centering of such measurements calls attention to a point that is very often overlooked. In any problem, after we have decided on the finest unit of measurement to be used, it is better to record any measurement not to the nearest position on the scale, but at that scale value at the beginning of the interval within which the measurement falls. Such a practice has the advantage of eliminating the necessity of estimating a mid-position between two scale readings and in no way handicaps the statistical treatment of the results. If such a method of recording results had been followed, the bars in the frequency distribution of Fig. 29 would run over the intervals 10-11, 11-12, etc., rather than over the intervals 9.5-10.5, 10.5-11.5, etc.

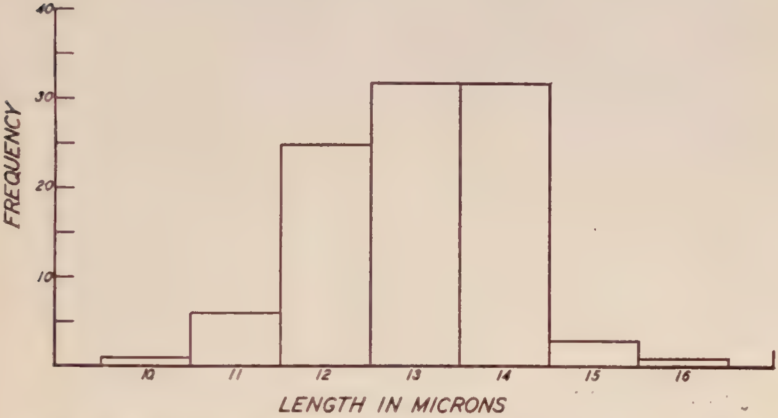

FIG. 29.—Frequency Distribution of Lengths of Trophozoites of *Giardia bradypi.*

Let us, then, define the statistical constants that are used to present these two ideas:

1. *Centering constants.* There are three centering constants in common use; they are the arithmetic mean, the median, and the mode, and are defined as follows:

Arithmetic mean or *average* of a set of measurements is the sum of their values divided by the number of such measurements.

Median is the value of that measurement above or below which one-half of the observations fall. It should be noted that if the number of measurements is even, the median is not definitely fixed by this definition, but may be any value between those two measurements which are the central ones in the series. It is usual in this case to take the average of the two central values.

Mode is the value of that measurement which would occur most frequently if an indefinitely large number of measurements were taken. In practice, the mode is a difficult constant to compute, for it should be determined as the modal position of the frequency curve that best fits the observations, rather than as the specific measurement in the series which is most frequent, and this involves the choice of a form of frequency curve, the fitting of such a curve and the determination of its mode.

2. *Constants of variation or dispersion.* The common constants of variation are: range, average deviation from the mean value without regard for sign, average positive deviation from the mean value, average negative deviation from the mean value, standard deviation, coefficient of variation, and quartile limits.

Range is the distance on the scale from the smallest measurement to the greatest, and although of interest and significance in a problem, it forms a poor constant by which to judge the variability of the material at hand, since it is dependent on only two of the measurements in the series and is, therefore, an unstable constant.

Average positive deviation and *average negative deviation* are, as their titles indicate, the averages of the positive and the negative deviations from the mean, taken separately.

Standard deviation is the square root of the average squared deviation from the mean. This measure of variation is the one that is in most common use in the field of statistics, although it is difficult for the beginner to see why this should be preferred to average deviation. It will be sufficient to say at this point that the reasons for its adoption in the field of statistics are:

a. That it is a comparatively stable constant, *i.e.,* relative to other measures of variation, it does not change much from one sample to another when these samples are taken from the same distribution.

b. It is the measure of variation that is of importance in the method of least squares and in the normal curve of errors.

c. Tables of distribution of frequency according to this measure of variation have been made up, and are available for use for those problems in which it may be assumed that the material is normally distributed.

Coefficient of variation is the ratio of the standard deviation to the mean value, multiplied by 100; that is, it is the variation measured in terms of standard deviation, stated as percentage of the mean.

There are two *quartile limits*, the upper and the lower; the lower quartile limit is that value below and above which one-fourth and three-fourths respectively of all measurements occur. The upper quartile limit is that value below and above which three-fourths and one-fourth respectively of all measurements occur. It will be seen that the quartile limits are measures of dispersion that are directly associated in idea with the median, and if the median is used as a centering constant, the quartile limits should be used to measure dispersion.

If the arithmetic mean is used as a centering constant, the dispersion should be measured in terms of standard deviation and coefficient of variation. It is well in any problem to state the range in order that the reader may have some idea as to the extremes of the variation.

The statistical constants used in connection with the frequency distribution may be found directly from the individual measurements by following the definitions given above, but experience with the computation of certain of these constants has enabled the statistician to reduce the arithmetic labor involved to a minimum, and it may be useful to indicate the method of determining these constants that has been found to be advantageous. For this purpose we shall compute the values of the arithmetic mean and standard deviation for the frequency distribution given above.

DETERMINATION OF THE MEAN AND STANDARD DEVIATION OF THE LENGTH OF TROPHOZOITES OF *Giardia Bradypi*

Length in microns	Frequency (f)	x	xf	x^2f
10	1	—2	—2	4
11	6	—1	—6	6
12	25	0	—	—
13	32	1	32	32
14	32	2	64	128
15	3	3	9	27
16	1	4	4	16
Total	100		101	213

If we use Σ to indicate summation, the following formulæ and procedure will be used in determining the mean and standard deviation:

$$N = \Sigma f = 100 \qquad \Sigma xf = 101 \qquad \Sigma x^2 f = 213$$

$$\nu_1 = \frac{\Sigma xf}{N} = \frac{101}{100} = 1.01$$

$$\nu_2 = \frac{\Sigma x^2 f}{N} = \frac{213}{100} = 2.13$$

$$\nu_1^2 = \underline{1.0201}$$

$$\mu_2 = \nu_2 - \nu_1^2 = 1.1099$$

$$\text{Standard deviation} = \sigma = \sqrt{\mu_2} = 1.0535$$

$$\text{Mean} = \text{origin} + \nu_1 = 12 + 1.01 = 13.01$$

While the above computation form is self-explanatory, it will perhaps be well to call attention to one or two points. The scale labeled x in the form above is arbitrary as to the choice of its origin. It really constitutes a new scale of length in microns with a O-point at twelve microns and with graduations of one micron above and below this O-point. In certain cases the unit chosen on the x-scale is not the exact unit on the original scale of measurement, but is some multiple of it. The reason for introducing this new scale is to cut down the arithmetic involved, and its origin and the size of its units are chosen so as to keep the x values as low as possible. In the case where the unit on the x scale is some multiple of the unit on the actual scale of length, it is necessary to change the mean and standard deviation back to the units of the original scale.

The terms ν_1 and ν_2 are called relative moments and together with higher moments such as $\nu_3 = \frac{\Sigma x^3 f}{N}$, $\nu_4 = \frac{\Sigma x^4 f}{N}$, etc., are common in statistical computation. The higher moments are used for the determination of statistical constants of more complex character,—such as the mode, skewness, etc., the determination of which will not be considered in this article but may be found in any text in the statistical method.

The arithmetic average is used so commonly as a centering point that it is perhaps unnecessary to discuss its properties, but it may be advisable to consider the possible interpretations of the standard deviation. By definition, standard deviation is the root mean squared deviation from the arithmetic mean, and may be used directly as an index of variation, the value of which will increase with experience in its use. Aside from its use as an index of variation, it has another value in connection with material that may be considered to be normally distributed, this value arising from the fact that the standard deviation is one of the important constants in the normal or Gaussian curve of errors. The form of this curve, a knowledge of which is necessary for an appreciation of standard deviation, is indicated in Fig. 30. As will be seen the standard deviation constitutes a unit of measure along the x axis and the area under this curve between any two positions on the x axis (such as A and B) represents the proportion of cases that would fall within these limits. Since the area under this curve is so frequently needed, tables have been prepared which give this area for any positions desired when these positions have been expressed in terms of standard deviation. A brief tabulation [2] of these areas is given in Table 2 and the table should be read as follows:

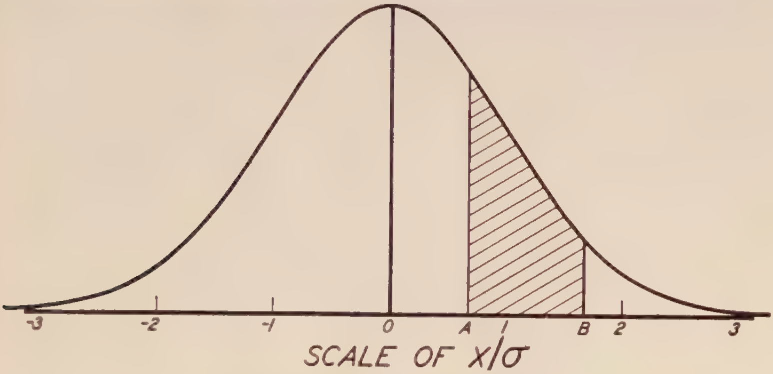

FIG. 30.—The Normal Curve.

Between the mean and a value of $\frac{x}{\sigma}$ of unity, thirty-four per cent of the area is included and this statement applied to the problem at hand indicates that between the mean length, 13.01 microns, and the length one standard deviation away from this, 14.06 microns, we would expect to find thirty-four per cent of our measurements in case the material is normally distributed. In Table 2 the area of the curve has been expressed not only in terms of standard deviation but in terms of a unit that is called probable error. Probable error may be briefly defined as that value on the x-axis which when scaled off on either side of the mean will include fifty per cent of the normal curve. Probable error is therefore directly related to standard deviation and either unit of variation may be used in any problem. The relationship between them is given by the expression

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[2] For a complete table of this sort, see Pearl, 1923.

$$P.\ E. = .67449\sigma$$

In the problem under consideration we would state that the standard deviation of the individual measurements is 1.05 microns

TABLE 2

TABLE OF AREAS OF THE NORMAL CURVE

$\frac{x}{\sigma}$	$\frac{x}{P.E.}$	Area from middle of curve $\left(\frac{x}{\sigma}=o\right)$ to indicated $\frac{x}{\sigma}$
.00	.00	.0000
.10	.15	.0398
.20	.30	.0793
.30	.45	.1179
.40	.59	.1554
.50	.74	.1915
.60	.89	.2257
.70	1.04	.2580
.80	1.19	.2881
.90	1.33	.3159
1.00	1.48	.3413
1.50	2.22	.4332
2.00	2.97	.4772
2.50	3.71	.4938
3.00	4.45	.4987
3.50	5.19	.4998

and that the probable error of the individual measurements is 0.71 micron. Adding this probable error to and subtracting it from the mean would give the statement that between the limits 12.30 microns and 13.82 microns, we would expect to find half of the individual measurements.

As a use of the table of areas of the normal curve, the following computation of the theoretical frequency of lengths of trophozoites is given:

TABLE 3

CALCULATION OF THE EXPECTED FREQUENCY OF MEASUREMENTS OF LENGTHS OF TROPHOZOITES OF *Giardia Bradypi*

Length in microns	Class limits	Deviations from mean (x)	Deviations from mean in terms of standard deviation $\left(\frac{x}{\sigma}\right)$	Area from mean to $\frac{x}{\sigma}$	Proportionate area between class limits	Theoretical frequency	Observed frequency
10 and under	—	—	—	—	.0086	0.86	1
11	10.5	—2.51	—2.383	.4914	.0673	6.73	6
12	11.5	—1.51	—1.433	.4241	.2383	23.83	25
13	12.5	—0.51	—0.484	.1858	.3648	36.48	32
14	13.5	0.49	0.465	.1790	.2423	24.23	32
15	14.5	1.49	1.414	.4213	.0697	6.97	3
16 and over	15.5	2.49	2.364	.4910	.0090	0.90	1
Total					1.0000	100.00	100

It will be seen that the theoretical frequency agrees satisfactorily with the observed and we may therefore consider that this material is normally distributed. A more thorough test of the agreement between the observed and theoretical frequencies will be given in the section on the Chi = square test.

It cannot be too strongly emphasized that the use of the normal curve in such cases is purely empirical, the curve being in no sense a "law of frequency." Its value rests rather on the fact that it does fit the distributions of measurements in many problems and due to the tables that have been prepared furnishes a very convenient form for the graduation of a distribution, to determine the proportion of cases that would be expected between any particular limits.

CORRELATION

Up to this point, we have attempted a description of measurements on a single variable, but problems often arise in which we take measurements on more than one variable, and it is necessary to consider their statistical description.

In the example used above, not only the length but the breadth of the trophozoites of *Giardia bradypi* was measured and a presentation of the measurements with regard to both length and breadth, along the same lines as the presentation as to length alone, will result in a frequency table in two dimensions such as is given in Table 4.

TABLE 4

CORRELATION OF LENGTH AND BREADTH OF TROPHOZOITES OF *Giardia Bradypi*

Length in Microns

		10	11	12	13	14	15	16	Total
Breadth in Microns	6	—	1	3	—	2	—	—	6
	7	1	5	18	21	5	—	—	50
	8	—	—	4	8	18	1	1	32
	9	—	—	—	3	7	2	—	12
Total		1	6	25	32	32	3	1	100

Such a table is called a correlation table, and is made up by entering in the cells of the table the frequencies with which the various combinations of length and breadth occur in the actual sample taken. For example, the frequency 21 in the table indicates that there were 21 trophozoites of *Giardia bradypi* having a breadth of seven microns and a length of thirteen microns. The totals at the bottom of the table and at the right hand side obviously give us the frequency distribution of the measurements of the two variables.

In biological material, tabulations of the type of the one above tend to take a form which indicates that measurements in the neighborhood of the center of the table are most frequent and that as we depart from the center in any direction the measurements become less frequent. A statistical description of such a table will necessitate the determination of a centering point for the table and a discussion of the way in which the points in the table deviate from such a center. The centering point used for such a table is that given by the mean values of the individual frequency curves, and can therefore be found by computing the means of the frequency distributions at the bottom and at the right-hand margin of the table. We would naturally expect to measure the variation of the measurements from this center by the use of the standard deviations of the frequency distributions in the margins, and this would be a proper procedure except for the fact that it is very common for such tables to show a trend. For example, in the table at hand, it can be seen on inspection that the shorter trophozoites tend to be narrower and the longer trophozoites tend to be broader. This tendency of one variable to change its value with a change in the value of the other variable introduces a concept not present in the treatment of a single variable, which is embraced under the general head of correlation.

Let us examine some of the ideas that enter into a proper concept of correlation. In the first place, in such a table, we should like to describe the trend of one variable with the other, and while this may be considered from many points of view, it is perhaps most clear when we think of the changes in the mean value of one variable that occur with changes in the other variable. For example, the average breadth of trophozoites eleven microns in length is clearly different from the average breadth of trophozoites fourteen microns in length. We might, therefore, very naturally ask the question as to how the mean breadth of the trophozoite increases as we step from one length class to another. Conversely, if we examine the table as to the average length of the trophozoites of the same breadth, we see that the broad trophozoites have a greater average length than the narrow ones, and we may therefore ask the question as to the increase in average length of the trophozoites that occurs as we pass from one breadth class to the next.

It is one of the functions of any study of correlation to answer these two questions, and in the case of normal correlation, the answer is given by determining two lines, one giving the movement of mean breadth with length and the other giving the movement of mean length with breadth. These two lines are called regression lines and their determination will be illustrated in the example which follows.

Another idea involved in the general subject of correlation arises from the fact that it is obvious on inspection that trophozoites of a specific length class, say twelve, are less variable in breadth than the total group of trophozoites. Similarly, trophozoites of a specific breadth class are clearly less variable as to length than trophozoites in general. Thus, in studying the correlation between two variables, an important point will be the determination of the reduction in the variability of one measurement that arises on selecting individuals that are constant as to the other measurement. This will be done by considering the standard deviations of one measurement for individuals that are constant as to the other measurement; for example, in the table at hand, we shall consider the standard deviation in breadth of the 25 individuals that have the length 12; similarly for the 32 individuals that have a length 13, etc. Conversely, we shall consider the standard deviation in length of the 50 individuals whose breadth is 7 and of the 32 individuals whose breadth is 8, etc. A comparison of these standard deviations within specific arrays of the table with the general standard deviation of the variables under consideration is another function of the study of correlation.

The determination of the regression lines and of the variability within the arrays of a correlation table leads in the case of normal correlation to an index number that is of considerable importance. This index number is called the correlation coefficient and its determination together with that of the other important elements of a correlation table will be illustrated in the following example:

DETERMINATION OF THE CORRELATION BETWEEN THE LENGTH AND BREADTH OF TROPHOZOITES OF *Giardia Bradypi*

Length in Microns

		10	11	12	13	14	15	16	Total	v	fy	fy^2	fx	fxy
Breadth in Microns	6	..	1	3	..	2	..	..	6	—2	—12	24	—3	6
	7	1	5	18	21	5	..	..	50	—1	—50	50	—26	26
	8	..	..	4	8	18	1	1	32	0	0	0	19	0
	9	..	..	..	3	7	2	..	12	1	12	12	11	11
	T't'l	1	6	25	32	32	3	1	100	..	—50	86	1	43
	x	—3	—2	—1	0	1	2	3	..	..	..	..	..	..
	fx	—3	—12	—25	0	32	6	3	1	..	..	..	..	..
	fx^2	9	24	25	0	32	12	9	111	..	..	..	..	..

$$\nu_{1x} = \frac{\Sigma fx}{N} = \frac{1}{100} = 0.01 \qquad \nu_{1y} = \frac{\Sigma fy}{N} = \frac{-50}{100} = 0.5$$

$$\nu_{2x} = \frac{\Sigma fx^2}{N} = \frac{111}{100} = 1.11 \qquad \nu_{2y} = \frac{\Sigma fy^2}{N} = \frac{86}{100} = 0.86$$

$$\mu_{2x} = \nu_{2x} - \nu_{1x}^2 = 1.1099 \qquad \mu_{2y} = \nu_{2y} - \nu_{1y}^2 = 0.61$$

$$\sigma_x = \sqrt{\mu_{2x}} = 1.0535 \qquad \sigma_y = \sqrt{\mu_{2y}} = 0.7810$$

$$\text{Mean of } x = m_x = 13. + 0.01 = 13.01$$

$$\text{Mean of } y = m_y = 8 - 0.5 = 7.5$$

$$\frac{\Sigma xy}{N} = \frac{43}{100} = 0.43$$

$$\nu_{1x} \,.\, \nu_{1y} = \underline{-0.005}$$

$$\text{Difference} = 0.435$$

$$\sigma_x \,.\, \sigma_y = 0.8228$$

$$r_{xy} = \frac{\dfrac{\Sigma xy}{N} - \nu_{1x}\nu_{1y}}{\sigma_x\sigma_y} = \frac{0.435}{0.8228} = 0.5287$$

$$(x - m_x) = r\frac{\sigma_x}{\sigma_y}(y - m_y)$$

$$x - 13.01 = 0.7132(y - 7.5)$$

$$x = 7.6610 + 0.7132y$$

$$(y - m_y) = r\frac{\sigma_y}{\sigma_y}(x - m_x)$$

$$y - 7.5 = 0.3919\ (x - 13.01)$$

$$y = 2.4014 + 0.3919x$$

$$\sigma_{x.y} = \sigma_x \sqrt{1 - r^2} = 0.8942$$

$$\sigma_{y.x} = \sigma_y \sqrt{1 - r^2} = 0.6629$$

Having determined the constants and equations that arise in the case of simple correlation, it would be well to look at the meaning of these constants.

The terms $m_x = 13.01$ and $m_y = 7.5$ indicate that the mean length and breadth of the trophozoites are respectively 13.01 and 7.5 microns.

The terms $\sigma_x = 1.05$ and $\sigma_y = .78$ measure in microns the variability of trophozoites as to length and breadth.

The equation

$$x = 0.6769y + 7.94$$

is used to determine the mean length of trophozoites that would be expected in trophozoites of specific breadth. For example, in considering a group of trophozoites all of breadth 8 microns, we would substitute 8 for y in the above equation and determine the value of x. This value, $x = 13.07$, states that the mean length of trophozoites of breadth 8 microns is 13.07 microns. Similarly, for any other breadth class.

In the same way the substitution of a specific value of x in the equation

$$y = 2.4014 + .3919x$$

will give us the mean breadth of trophozoites of any specific length class.

The term $\sigma_{x.y}$ represents the variability in length of trophozoites of given breadth and the term $\sigma_{y.x}$ represents the variability in breadth of trophozoites of given length.

The correlation coefficient, r, is an index number of the degree of association between the two measurements in question, and may take values only between -1 and $+1$. When r is negative, it means that one variable is increasing as the other decreases, while when r is positive, it means that the two variables increase together. In the present example, the positive value of r indicates that the longer trophozoites are also the broader trophozoites. A value of $r = 0$ indicates no association between two variables, and in this case, $\sigma_{x.y}$ is equal to σ_x, and $\sigma_{y.x}$ is equal to σ_y, so that the variability within the arrays is the same as the variability in the marginal columns. When r is either $+1$ or -1, the correlation is perfect, which means that any specific value of one variable is always associated with a fixed value of the other variable. The frequencies in the correlation table always fall in a straight line in this case.

PROBABLE ERROR CONCEPT

In the two previous sections we have been discussing certain statistical constants that have been found useful in evaluating quantitative material. One of the points that this discussion has emphasized is the variability of the individual measurements themselves, and we have learned how to evaluate this variability and make use of it to express the proportions of individuals we would expect to find within given class intervals. An acknowledgment of the fact that individuals are subject to variation leads to the additional thought that this variability of the individuals will have an effect on their arithmetic mean or on any statistical constant that may be derived from their individual measurements. A discussion of this effect is embraced under the heading of probable error concept, and it may be summarized as follows:

Whenever we measure a group of individuals and compute averages from such measurements, we are interested not in the particular sample under consideration but in the general universe of like organisms from which this sample was drawn, and we hope to use this sample as an indication of what will be found in the universe. If, however, as we have seen to be the case, the individuals of the universe at hand are themselves variable as to thus raise the following question: If many samples, each sample drawn will not necessarily be equal to the mean value of the universe, but will vary from the mean value of the universe by some amount that is a function of the individual variability. We thus raise the following question—If many samples, each sample of size N, were drawn from a given universe and for each of these samples a mean value was computed, how variable would these means be? In an attempt to answer this question, the first important result brought out by the statistical method is that means of samples of the type described above will tend to distribute themselves in a normal form regardless of the form of distribution of the individuals, and the standard deviation of the normal curve under which these means tend to fall will constitute a natural measure of their variability. This standard deviation of the means (σ_m), is, as would be expected, a function of two factors, namely the variability of the individuals, σ, and the size of the sample, N; the formula expressing the standard deviation of the mean is

$$\sigma_m = \frac{\sigma}{\sqrt{N}}$$

As in the case of the individuals, the probable error of the mean can be found by multiplying the standard deviation of the mean by .67449, so that the formula for probable error of the mean will read

$$\text{P. E.}_m = .67449 \frac{\sigma}{\sqrt{N}}$$

The value obtained by using this formula will set up limits such that it is an even chance that the mean value of the universe from which this sample is taken lies within the interval laid off by adding this probable error to and subtracting this probable error from the mean value of the sample. The likelihood that the mean value of the universe lies within limits set up by any multiple of the probable error will be found by referring to Table 2. Thus, having the facts for a sample, we are able to form some judgment as to the mean value of the unknown universe from which this sample was drawn.

The discussion given for the probable error of the mean is in general paralleled when we consider the probable error of any statistical constant. We find that such constants have variabilities that are functions of the variability of the individuals and of the size of the sample. The following set of equations presents the probable errors of the more important of the statistical constants:

TABLE 5

TABLE OF PROBABLE ERROR FORMULÆ

Constant	Probable Error
Arithmetic Mean	$\pm .67449 \frac{\sigma}{\sqrt{N}}$
Median	$\pm 1.25332 \times P.E.$ mean
Standard Deviation	$\pm .67449 \frac{\sigma}{\sqrt{2N}}$
Coefficient of Variation	$\pm .67449 \frac{C.V.}{\sqrt{2N}} \left\{ 1 + 2 \left(\frac{C.V.}{100} \right)^2 \right\}^{\frac{1}{2}}$
Correlation Coefficient	$\pm .67449 \frac{1 - IV}{\sqrt{N}}$

The knowledge of any constant and its probable error suffices to tell us the value and variability of the attribute under discussion, but in many cases, we wish to consider the value of a given attribute in comparison with the value of the same attribute for a different sample. For instance, we may have determined the mean value of a certain measurement for an experimental group and the mean value of the same quantity for a control group, and we immediately ask the question—does this mean for the experimental group differ from the mean for the control group? Obviously, this question is not limited to a mere comparison of the arithmetic values of these means, but implies a discussion of the question as to whether or not the difference of these means is a significant one when we consider variability of the individuals in the two samples involved. To answer this question, we shall need to take the difference between the means in question and consider this difference in the light of the probable error of the difference. Thus, we feel the need for a formula to represent the probable error of the difference between any two quantities. This formula is given by

$$P.E.\text{ of }(x-y)=\sqrt{(P.E.\text{ of }x)^2+(P.E.\text{ of }y)^2}\,[3]$$

As an illustration of the use of this equation, let us consider the following distribution of the number of spines for parents and offspring in a family of *Difflugia corona.*

No. of Spines	Frequency in Parent	Frequency in Offspring
2	4	4
3	8	14
4	30	31
5	13	11
6	12	7
7 and over	2	2
Total	69	69

For parents we have a mean number of spines of 4.391 with a probable error of 0.094 and for offspring a mean of 4.145 with a probable error of 0.094.

The difference between these two means is 0.246 and the probable error of this difference is given by

$$P.E._{\text{diff.}}=\sqrt{P.E._{1}^{2}+P.E._{2}^{2}}=\sqrt{(.094)^2+(.094)^2}=0.133$$

Therefore

$$\frac{Diff.}{P.E._{\text{diff.}}}=\frac{0.246}{0.133}=1.85$$

Referring to Table 2, we find that the area from the center of the normal curve to an $\frac{x}{P.E.}$ of 1.85 is 0.387. Thus, we may state that the chance of the occurrence of a difference as large as or larger than the one observed is 0.5 — 0.387 or 0.113. Since we have no *a priori* knowledge as to whether or not the parent or the offspring has the greater number of spines, we should allow for the possibility of this difference being in either direction by using twice 0.113 or 0.226. Thus we may say that if we repeat the above experiment many times we would expect as large a difference as did occur, to appear about 23 times in 100 even though there were no real difference between the number of spines in parent and offspring in the general universe.

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[3] This is the most frequently used equation of a general set of formulæ giving means and standard deviations for simple functions such as sums, differences, products and quotients of one or more variables. These general equations will be found in references Dunn (1929) and Pearl (1923).

By the procedure above outlined, we may determine the significance to be attached to differences between any two statistical constants.

CHI-SQUARE TEST

In addition to questioning the significance of the difference between any two statistical constants, we often question significance in a more general form. We consider the distributions of measurements that we wish to compare and ask the general question as to whether or not they are samples that might very probably have come from the same distribution, the idea involved being that if we find that it is quite probable that they might have arisen from the same distribution we would attach no significance to any of the differences that may exist between these distributions. If, on the other hand, we find that they are samples that would be very unlikely to come from the same universe, we will attach significance to their differences. To answer this question, a statistical method called the Chi-square test has been developed. This method depends upon the derivation of a general variation constant, χ^2, and the relationship between this constant and the probability that such differences as were observed might have arisen by sampling alone. The use of this test may perhaps be made clear by an example and we shall apply this test to see whether or not the general distributions of parent and offspring of *Difflugia corona* as to number of spines are significantly different.

CHI-SQUARE TEST

NUMBER OF SPINES OF PARENT AND OFFSPRING OF *Difflugia Corona*

	Number of Spines						
	2	3	4	5	6	7 and over	Total
Parent (1)	4	8	30	13	12	2	69
Offspring (2)	4	14	31	11	7	2	69
(1)+(2) (3)	8	22	61	24	19	4	138
(1)/69 (4)	.0580	.1159	.4348	.1884	.1739	.0290	1.0000
(2)/69 (5)	.0580	.2029	.4493	.1594	.1014	.0290	1.0000
(4)—(5) (6)	0	—.0870	—.0145	.0290	.0725	0	—
Sq.of (6) (7)	0	.007569	.000210	.000841	.005256	0	—
(7)/(3) (8)	0	.00034405	.00000344	.00003504	.00027663	0	.00065916

Chi-square $= \chi^2 = 69 \times 69 \times .0065916 = 3.13826$

With the value of χ^2, we go to Pearson's *Table for Statisticians and Biometricians*, page 26, and we look in the column headed $n' = 6$, this being the number of classes for which we are making our comparison. Within this column we interpolate for a value of P to correspond to a value of $\chi^2 = 3.138$. For this value, we find 0.679. The interpretation of this probability is as follows: If there were no difference in the number of spines of parent and offspring and we repeated the above sampling process many times, we would expect to get samples as different as or more different than the ones that did appear, 68 times in 100. Thus, we may conclude that in the present case there is no reason for assuming that parent and offspring differ as to the number of their spines. If P had been small, we would have concluded that the difference was significant. In general, it is dangerous to assume that differences are significant unless P is as small as 0.01.

The χ^2 test has an entirely different method of computation when we wish to compare a sample with a theoretical distribution. As an illustration of this, consider the distribution of length of trophozoites in comparison with the theoretical distribution as given in the section on frequency distribution. The computation for χ^2 in this case is as follows:

COMPARISON OF OBSERVED AND THEORETICAL DISTRIBUTIONS OF LENGTH OF TROPHOZOITES OF *Giardia Bradypi*

	Length in Microns					
	11 and under	12	13	14	15 and over	Total
Theoretical (1)	7.59	23.83	36.48	24.23	7.87	100.00
Observed (2)	7.00	25.00	32.00	32.00	4.00	100.00
(1)—(2) (3)	0.59	—1.17	4.48	—7.77	3.87	—
Sq. of (3) (4)	0.3481	1.3689	20.0704	60.3729	14.9769	—
(4)/(1) (5)	0.0459	0.0574	0.5502	2.4917	1.9030	5.0482

$$\chi^2 = 5.0482$$

With this value of χ^2, a value of P is found as in the preceding example. This value of P is .283, and indicates that in sampling from a normal distribution we would get as large discrepancies as did occur in this sample, 28 times in 100. Thus we may state that the distribution is essentially normal.

RATE CURVES

Under the heading of correlation we discussed the method of expressing a relationship between variables in the case where the relationship took on a linear form; that is, in the case where a change of a unit in one of the variables was accompanied by a constant change in the other variable. There are of course many cases in which the variables under consideration are not thus related, but a general discussion of non-linear correlation is beyond the scope of this article. It will, however, be advisable for us to consider one non-linear case due to its importance in expressing rates of reaction. The case in question is that of geometric increase or decrease, or the curve of constant proportional increase as contrasted with that of constant absolute increase treated under the heading of correlation.

If the two variables under discussion are represented by x and y, we say that y is changing geometrically with x when the two variables are following an equation of the form

$$y = 10^{a+bx} = A(B)^x$$

in which equation $A = 10^a$, and $B = 10^b$. By taking logarithms of both sides of this equation, we may write

$$log\ y = a + bx$$

and this equation shows that the two variables x and $log\ y$ are related in a linear fashion. This fact has led to the development of a special form of graph paper on which one scale used for x is arithmetic and the other scale used for y is logarithmic. Any set of observations that fall along a straight line when plotted on such paper will follow the equation of geometric increase.

The mode of derivation and the significance of the constants of the equation will be given in the following example:

In an article by Hartman (1927) is given a set of measurements of the average area in square microns of twenty *Plasmodia præcox* in the asexual stage, taken at approximately hourly intervals from 7.30 P.M. of one day until 4.00 P.M. of the following day. These parasites change in size as is indicated in the following table:

TABLE 6

GROWTH OF ASEXUAL PARASITE

Time of day	x	Average area in square microns of 20 parasites y
7.30 P.M.	−5	1.26
8.00 “	0	1.00
9.00 “	1	1.37
10.00 “	2	1.34
11.00 “	3	1.63
12.00 M.	4	2.47
1.15 A.M.	5.25	2.81
2.00 “	6	2.72
4.00 “	8	2.49
6.00 “	10	4.37
7.00 “	11	5.88
8.00 “	12	6.48
9.00 “	13	7.14
10.00 “	14	8.72
11.00 “	15	13.21
12.00 M.	16	10.66
1.00 P.M.	17	12.52
2.00 “	18	16.12
3.00 “	19	17.53
4.00 “	20	19.78

If we plot these observations on ordinary arithmetic paper as in Fig. 31, we note at once that an increase in size is not constant for a given length of time but tends rather to be larger for the later periods. If, however, we plot these observations on arith-log paper as in Fig. 32, we see that the points tend to take a linear position. This indicates that while the absolute rate of change of these parasites is variable, the proportional rate of change is practically constant. We may, therefore, fit to these points an equation of the type

$$y = 10^{a+bx}$$

This may be done in several ways, one method being to take the logs of the y's and relate these logs to their corresponding x values by the method discussed under the head of correlation. In this case, however, it will be sufficient to fit a line by the method of inspection and the line shown in Figure 32 was drawn in the position that seemed best to represent the points. After the line is drawn, two values may be read from the line as follows:

$x = 0$ (time 8 P.M.) $y = 1.14$
$x = 20$ (time 4 P.M.) $y = 20$

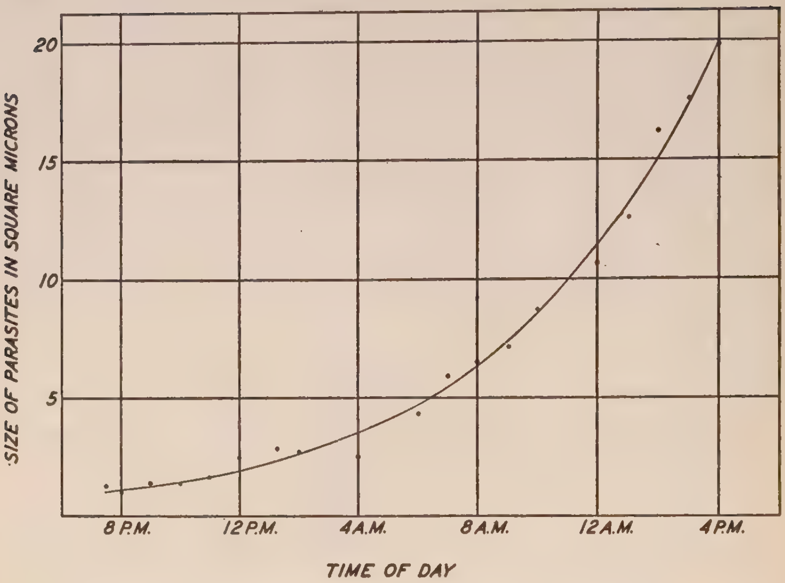

FIG. 31.—Growth of *Plasmodium præcox* in the asexual stage. (Arithmetic scale.)

Substituting these values in the general equation

$$\log y = a + bx$$

and solving for a and b we have

$$\log\ 1.14 = .0569049 = a$$
$$\log 20 = 1.3010300 = a + 20b$$
$$a = .0569049$$
$$20b = 1.2441251$$
$$b = .0622063$$

By considering the fact that

$\log A = a$ and

$\log B = b$ we find that

$A = 1.14$ and

$B = 1.1540$

Thus we may write the equations as

$$y = 10.^{.0569+.06224x} = 1.14\ (1.154)^x$$

The important constant in this series is B, since it tells us that the average size of these parasites at any time is 1.154 times as great as the size an hour earlier; thus we may say that these organisms increase at a rate of fifteen per cent per hour.

In any case in which it is desirable to express the rate of change of one variable with respect to another, and these variables plot on arith-log paper in a linear fashion, this procedure may be used. It should be noted that if y is declining as x increases, the constant B will be less than 1. A value of B such as 0.96 would indicate that a four per cent decrease in the y variable accompanies a unit change of x.

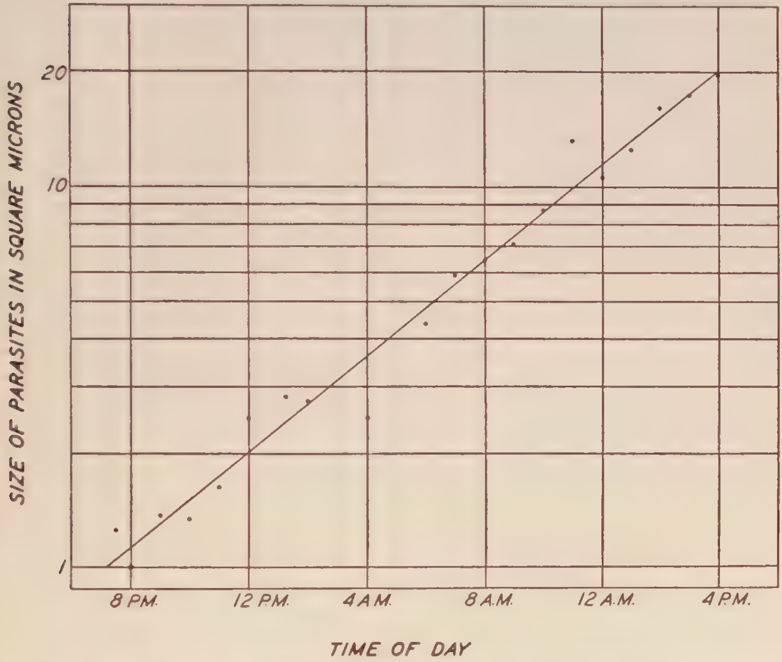

FIG. 32.—Growth of *Plasmodium præcox* in the asexual stage. (Arith-log scale.)

In many problems where an analytic expression of the rate is not desired, the arith-log paper will still be found of great convenience in discussing representative rates of change. As an illustration of the use of arith-log paper in such connection, see the paper by Andrews and Sanders (1928).

SUMMARY

The present paper is the briefest possible outline of certain elementary statistical procedures and may be found satisfactory as an introduction to statistical method. Any worker in the field of protozoölogy who attempts to apply statistical treatment to his material will find need for a more complete treatment of the points here introduced, and for a discussion of other phases of the statistical method. For such, refer to the textbooks on the statistical method by the following authors: Fisher (1925), Pearl (1923), and Yule (1927).

CHAPTER XLII

STANDARD METHODS AND REAGENTS

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METHODS

Descriptions of the following methods, most of which are standard in laboratories of parasitic protozoölogy, are presented in as reduced form as possible. More comprehensive treatments are to be found in the following books on microscopic technique to which the reader is referred: *Animal Micrology* (Chicago) by M. F. Guyer, *The Microtomist's Vade-Mecum* (Philadelphia) by A. B. Lee, *Pathological Technique* (Philadelphia) by F. B. Mallory and J. H. Wright, and *Microscopical Technique*[1] (New York) edited by C. E. McClung. For methods of obtaining blood from animals and of making various kinds of parenteral injections into animals see Chapters II and III, respectively, of Kolmer's *Infection, Immunity and Biologic Therapy* (Philadelphia). More detailed accounts of standard and specialized techniques of protozoölogical interest are to be found in *Parasitic Protozoa of Man* (Philadelphia) by C. F. Craig, and Vol. II of *Protozoology* (New York) by C. M. Wenyon.

Observation of Living Protozoa

In General. Living protozoa may be observed either with the brightfield or darkfield microscope. In the first case the specimen, if it be fecal, is emulsified in a sodium chloride solution of from 0.4 = 0.85 per cent; a drop of the emulsion is placed on a clean slide and is covered with a clean coverslip. The thickness of the smear should be such that it is just possible to read newspaper print through it. It is advisable, particularly when examining intestinal amœbæ, to provide for maintaining the specimen at 37° C. or thereabouts by the use of a warm chamber to enclose the microscope or a warm stage. If either of these two items of apparatus are not available, the temperature of the slide may be kept at a satisfactory height by placing heated coins upon it.

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[1] See particularly Chapter VIII on *Protozoological Methods* by D. H. Wenrich.

Fresh blood is simply diluted with an isotonic fluid such as physiological saline,[2] Ringer's,[3] or Locke's [4] solution to the point where the organisms present will not be obscured by the concentration of red blood cells. A drop of the diluted blood is placed upon a clean slide and covered with a clean coverslip.

Observation with the darkfield microscope is useful in counting flagella, observing ciliation, etc. Material to be examined must be as nearly free as possible from other small particles such as bacteria to reduce "flashing." Rapidly moving protozoa may be immobilized by holding the uncovered smear face downward over a bottle of ten per cent acetic acid for one minute.

Concentration Methods. Various concentration methods have been devised for the purposes of (1) facilitating the diagnosis of infections with intestinal amœbæ, flagellates, or coccidia, or (2) of providing a sufficient quantity of comparatively rare material for study or inoculation. These methods are applicable only to cyst-producing organisms, as the measures involved are, as a rule, too vigorous to permit the survival of anything but cysts.

[2] *Physiological saline*

Sodium chloride	8.5 gms.
Distilled water	1000.0 c.c.

[3] *Ringer's solution*

Sodium chloride	8.00 gms.
Calcium chloride (anhydrous)	0.20 gm.
Potassium chloride	0.20 gm.
Sodium bicarbonate	0.20 gm.
Dextrose (may be omitted)	1.00 gm.
Distilled water	1000.00 c.c.

[4] *Locke's solution*

Sodium chloride	9.00 gms.
Calcium chloride (anhydrous)	0.24 gm.
Potassium chloride	0.42 gm.
Potassium bicarbonate	0.20 gm.
Dextrose (may be omitted)	1.00 to 2.50 gms.
Distilled water	1000.00 c.c.

Simple sedimentation in a tall cylinder is commonly employed for the detection of all cyst-producing forms. The technique may be refined as described in Chapter XXXI (p. 281).

The following method, adapted from Yorke and Adams (1926), was devised for use with *Endamœba histolytica* but it would probably be equally useful for the other intestinal amœbæ and flagellates that produce cysts. The specific gravity of the sugar solution is not sufficiently high to carry coccidial oöcysts to the surface in any great quantity. One of the advantages of this method is that the cysts are recovered in a viable state and can be used for inoculation, culture, and various other purposes. Procedure is as follows:

A mass of feces is mixed with a little water until it is thoroughly emulsified. Shake the emulsion with from 500 to 1000 c.c. of water, pour into a tall glass cylinder and allow it to stand about fifteen minutes. Skim off the flotage with a wire loop covered with cheesecloth. Pour off the supernatant fluid and save it. Discard the lowest inch with the sediment.

Either centrifuge the supernate or allow it to stand overnight in a tall glass cylinder. In either case the sediment is shaken up thoroughly with a solution of cane sugar in water, of a specific gravity of about 1080 (20 grams of sugar in 100 c.c. of solution) and is centrifuged at high speed for two or three minutes. The upper one-fifth of the supernate is pipetted into a clean centrifuge tube, and the volume is made up to nearly capacity with water and is again centrifuged at high speed. The resulting sediment is examined for cysts.

The next method, adapted from Boeck's (1917) modification of the Cropper and Row (1917) technique gives satisfactory results with any kind of intestinal protozoan cyst. It is as follows:

At least 1 gram of feces is placed in 30 c.c. of normal saline and is stirred with the soda fountain mixer (or is thoroughly emulsified by some other more convenient method such as shaking with glass beads or shot) for from 2 to 10 minutes depending upon the consistency of the stool. When the specimen appears to be uniformly emulsified, 5 c.c. of ether is added and the resulting mixture is stirred or shaken for an additional 2 to 3 minutes.

> *Caution:* 1. If obliged to shake the ether mixture in a closed vessel, be careful to reduce the internal pressure at frequent intervals by opening the container!
>
> 2. Keep away from any open flame while shaking this mixture or releasing the gases from it!

The emulsion should be quickly placed in a separatory funnel, and allowed to stand for from 5 to 7 minutes. (Unless the soda fountain mixer is used to make the ether mixture, the mixing can be most easily accomplished in the separatory funnel.) After the required time, about 15 c.c. of the material is withdrawn from the bottom of the separatory funnel into a centrifuge tube, and is centrifugalized for 3 minutes. The supernate is decanted and the sediment is examined for cysts.

The concentration method of De Rivas is said to be very efficient for the cysts of intestinal protozoa. It is carried out as follows:

A small quantity (one or two grams) of feces is added to from five to ten cubic centimeters of five per cent acetic acid in a test tube and is thoroughly shaken. After the mixture is homogeneous it is filtered through several thicknesses of cheesecloth and five cubic centimeters of the filtrate is added to an equal quantity of ether in a fifteen cubic centimeter centrifuge tube. The tube is closed and is shaken horizontally, and then is centrifuged for a few minutes. The contents of the tube separate into four layers: the etherial extract at the top, the detritus plug near the middle, next the acetic acid solution, and lastly the sediment. The sediment is examined for cysts.

Other methods for concentrating coccidial oöcysts are given in Chapter XXXI.

Observation of Killed and Fixed Protozoa

Killed protozoa may be examined in smears or films or in cut sections.

Smears. Intestinal material may be smeared upon glass slides as indicated above. If a permanent preparation is not required the smear may be made in a solution containing iodine (one per cent iodine in two per cent potassium iodide) which will tend to render nuclei and flagella a trifle more conspicuous than they appear in a fresh living preparation.

If permanent preparations are required the smear may be "fixed" and stained. "Fixing" the organisms involves the precipitation of their protein constituents and is accomplished by immersing the slide or coverslip upon which the smear is made in one of such fluids as Schaudinn's,[5] Bouin's,[6] Carnoy's,[7]

[5] *Schaudinn's solution*

Saturated aqueous solution of mercuric bichloride	65 parts
95% ethyl alcohol	33 parts
Glacial acetic acid	2 to 5 parts

[6] *Bouin's solution*

Saturated aqueous solution of picric acid	75 parts
Formalin	25 parts
Glacial acetic acid	5 parts

[7] *Carnoy's solution*

Glacial acetic acid	1 part
Absolute alcohol	6 parts
Chloroform	3 parts

Flemming's,[8] or Zenker's [9] solutions. Schaudinn's solution warmed to a temperature of about 50° C. is the most popular fixative. The smear is left in the fixing fluid for about ten minutes and is then stained.

The iron-alum-hematoxylin method of Heidenhain, or some one of its numerous modifications, is the staining method of choice for protozoölogical material. The following procedures are routine in this laboratory for the staining of intestinal protozoa in general.

Smears are fixed as above in Schaudinn's fluid, after which they must not be allowed to dry during the subsequent transfers. The fixed smears are placed in 95 per cent alcohol to harden. This requires about five minutes after which they are washed for five minutes in tap water and placed in a 4 per cent solution of ferric alum for ten minutes. They are then rinsed off with tap water and placed immediately in the stain which is a 0.5 per cent solution of hematoxylin in water. From ten to twenty minutes is usually a sufficiently long time to stain the organisms. After they have been taken out of the stain they are again rinsed in tap water and decolorized in a 2 per cent solution of ferric alum. This is the most critical procedure of the entire process. Until the action of a particular combination of stain and decolorizer has been ascertained the progress of the destaining should be frequently checked by observing the slide (washed previously in tap water to temporarily check the decolorizing) under the microscope. After a few experiences with the material in hand it will be possible to time the exposure to the destaining fluid so that it will not be necessary to observe the slides under the microscope. When the contrast of cytoplasm and nuclear material is sufficiently great the process of destaining is brought to an end by washing thoroughly with tap water. If the slide is to be kept for a long time it is best to wash it for from two to several hours but if it is made for the purpose of immediate diagnosis ten minutes is sufficient time. After washing, the slide is immersed in 95 per cent alcohol for from two to four minutes, in absolute ethyl alcohol for five minutes, and in xylol for five minutes, after which a clean coverslip is mounted over the preparation with neutral balsam.

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[8] *Flemming's solution* (strong)

Chromic acid (1% aqueous solution)	15 parts
Osmic acid (2% aqueous solution)	4 parts
Add just before use:	
Glacial acetic acid	1 part

[9] *Zenker's solution*

Potassium bichromate	2.5 gms.
Mercuric bichloride	5.0 gms.
Sodium sulphate	1.0 gm.
Distilled water	100.0 c.c.
Add just before use:	
Glacial acetic acid	5.0 c.c.

The above method is recommended for purposes of diagnosis only. If material is to be prepared for careful cytological study the original method of Heidenhain should be more closely adhered to, allowing from four to twelve hours' immersion in the mordant (4 per cent ferric alum solution) and a similar length of time in the stain. By doing this a much more critical decolorization can be obtained. Sections may be stained in general in accordance with the directions above except that the paraffin will have to be removed either with xylol or chloroform before the staining is undertaken. In general the process will go somewhat more slowly with the sections than with the smears due to a greater thickness of the former.

Another method preferred by some protozoölogists for routine work is Mayer's hemalum staining technique. Procedure is as follows:

> The stock solution of Mayer's hemalum[10] is diluted at least twenty times, and the fixed smears are left in it for several hours or overnight. After staining, the films are left in running tap-water, or in slightly alkalinized distilled water until they are blue. They are then dehydrated in alcohol, cleared in xylol, and mounted in acid-free balsam. If the slides are overstained, they may be decolorized in acid alcohol,[11] washed, and reneutralized in running tap water.

A technique which, it is alleged, is a specific test for chromatin is becoming widely used by protozoölogists. It has been devised by Feulgen (Cowdry, 1928) and depends upon the presence of thymonucleic acid. The procedures are as follows:

1. Fixation.

Corrosive sublimate 6 per cent aq. sol.	98 parts
Glacial acetic acid	2 parts

[10] *Mayer's hemalum*

Hematein	1.0 gm.
Ethyl alcohol (90%)	50.0 c.c.
Alum	50.0 gms.
Distilled water	1000.0 c.c.

The hematein is dissolved in the alcohol with the aid of heat, and is added to the water in which the alum has been dissolved. Keep a crystal of thymol or some other fungicide in the stock solution.

[11] *Acid alcohol*

Ethyl alcohol (60%)	99.0 c.c.
Hydrochloric acid	1.0 c.c.

2. Hydrolysis.

Sections are exposed for two minutes to the action of cold dilute hydrochloric acid, followed by digestion for four to fifteen minutes in the same strength of acid at a temperature of 60° C.

Hydrochloric acid s.g. 1.19	82.5 c.c.
Distilled water	1000.0 c.c.

3. Rinse in cold dilute hydrochloric acid.
4. Rinse in distilled water.
5. Stain in fuchsin-sulphurous acid ninety seconds.

Basic fuchsin	1 gm.
Distilled water	200 c.c.

Boil, cool to 50° C., filter, add 20 c.c. of the dilute hydrochloric acid. When further cooled to 25° C., add 1 gm. of anhydrous sodium sulphite. When the solution is decolorized it is ready for use and should be kept in the dark.

6. Pass through 3 baths of dilute sulphurous acid.

Distilled water	200 c.c.
10 per cent aq. sol. of sodium sulphite	10 c.c.
Dilute hydrochloric acid	10 c.c.

7. Rinse in distilled water.
8. Counterstain in light green.
9. Mount in damar.

To make permanent preparations of hemaflagellates, smears of blood or tissue are made as follows:

A clean slide is laid upon a flat surface; a drop of blood is placed near one end and another slide (the spreader) is held between the thumb and middle finger at an angle of about 30° and is brought back until the blood is touched with the spreader and is distributed along its edge. The spreader is now pushed to the opposite end of the first slide so that the drop of blood is distributed evenly along the surface of the slide. Smears of tissue such as liver or spleen are made by lightly dabbing the slide with the cut surface of the tissue. Smears of blood or of tissue may be allowed to dry, after which they may be stained with Wright's, Leishman's or other modifications of Romanowsky stains, with the exception of Giemsa, in which case they should be previously fixed with absolute methyl alcohol.

In using Wright's, Leishman's, Wilson's, Hasting's or any of these similar preparations which are dissolved in a fixative (absolute methyl alcohol) the film is covered with the stock stain for from forty-five seconds to two minutes, after which distilled water [12] is added drop by drop until a metallic scum appears upon the surface of the mixture. This diluted solution is allowed to remain for from two to four minutes. At the end of this period, the slide is rinsed in distilled water and is dried in air. If overstained, it may be held in running water for a few minutes. A precipitate of stain may be removed by immersing the slide for an instant in a jar of the stock stain. This will dissolve the precipitate without decolorizing the stain. Inasmuch as different batches of stain frequently behave very differently on various kinds of organisms, the time intervals given above will have to be determined for each stain with each species of protozoa stained.

In using Giemsa's stain the film is fixed with any of the ordinary fixatives (absolute methyl alcohol is most commonly employed) and is then left in a one in ten dilution of the stock stain in tap water [13] for from ten minutes to two hours depending upon the material and particular batch of stain. Overstained films can be nicely decolorized by holding them in running tap water for a few moments. The film is dried in air.[14]

See Chapters XXVIII and XXXV for special uses of the Romanowsky stains on plant flagellates and malarial parasites.

Sections. Tissue containing protozoa may be sectioned following the standard histological procedures for that technique. These processes are so well known that they are not given in detail here. Briefly, the material must first be "fixed" and hardened, washed to remove the excess fixing agent, dehydrated in alcohol, cleared

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[12] The pH of the diluent should be within the range of 6.0 to 6.6. A suitable buffered solution for dilution purpose is given below. It has a pH of 6.4.

KH_2PO_4	6.63 gms.
Na_2HPO_4	2.56 gms.
Distilled water	1000.00 c.c.

[13] If the local supply of tap water is for one reason or another unsuitable for this purpose, distilled water which has been slightly alkalinized by standing for some time with a small quantity of calcium carbonate is quite satisfactory.

[14] If possible, when drying films which have been stained by some one of the Romanowsky techniques, an air blast should be used in order to dry the film as quickly and uniformly as possible. It not infrequently happens that in a film which has been left flat on the table or even tilted on edge to drain, areas of differing staining intensities are apparent when the slide has dried. The reason for that is that the drying has not been uniform, and that decolorization has progressed further in one area than in another.

in xylol or chloroform, infiltrated, and finally embedded in paraffin. Rarely the material is embedded in celloidin.

The staining of sectioned tissue containing protozoa does not differ essentially from the staining of smears except that the sections are relatively much thicker than the smears so that longer intervals of time must be allowed for such procedures as the replacement of water with alcohol or *vice versa*, impregnation with the mordant or the stain, and clearing. Sections to be used for pathological study are usually stained with Delafield's hematoxylin[15] and eosin. For cytological details, particularly of the parasites, use Heidenhain's iron-alum-hematoxylin or Mayer's hemalum. In studying tissues which contain blood-inhabiting protozoa it is many times worth while to stain a few sections with Giemsa's stain in order to get the Romanowsky effect. The slides are passed through descending grades of alcohol to water and are stained for from one to twelve hours in freshly diluted (one in ten) Giemsa's stain. Do not pass the slides through the alcohols to return to xylol as the stain will be extracted. Use a number of mixtures of xylol and acetone (5:95, 30:70, 50:50) before going into pure xylol.

Cultivation Methods

Amœbæ. The cultivation methods which are in general use for *E. histolytica* are treated in Chapter XIX. These methods appear to be suitable, with at most only slight modifications, for the cultivation of other entozoic amœbæ of man and lower animals. In this connection see the papers of Barret and Smith (1924 and 1926), Chiang (1925), Dobell (1926), Drbohlav (1925, 1925*a*,

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[15] *Delafield's hematoxylin*

Hematoxylin (crystals)	4.0 gms.
Ethyl alcohol (95%)	25.0 c.c.
Dissolve hematoxylin in alcohol and add, drop by drop to	
Ammonia alum (saturated solution)	400.0 c.c.
Expose to light and air in an unstoppered bottle for three or four days. Filter, and add:	
Methyl alcohol (90%)	100.0 c.c.
Glycerin	100.0 c.c.

Allow solution to stand uncorked in the light until it darkens in color and is "ripe" (Hematoxylin oxidized to hematin).

1925*b*), Das Gupta (1925), Howitt (1925), and Thomson and Robertson (1925).

Intestinal flagellates. The cultivation of intestinal flagellates and various media employed have been discussed at some length in Chapters VII and XVI.

Hemoflagellates. All of the *Leishmania* organisms and some species of *Trypanosoma* have been grown on the blood-agar medium devised by Novy and MacNeal (1903) modified by Nicolle (1908) and known to protozoölogists as N. N. N. medium.

The agar base has the following ingredients:

Agar	14 gms.
Sodium chloride	6 gms.
Distilled water	900 c.c.

This mixture is tubed while hot in five cubic centimeter quantities and is autoclaved. Before use, from two to five cubic centimeters of fresh rabbit blood (most easily obtained by cardiac puncture using aseptic technique) are added to each tube which has been heated to about 50° C. The tube is rapidly rolled between the two hands to mix the warm agar and blood. Vibration of the tubes during the rotating procedure must be avoided so that the mixture will be free from bubbles. The tubes are tilted sufficiently so that the contents will harden into slants. They are then incubated at 37° C. for twenty-four hours to test for sterility. In using the medium, a small quantity of blood or tissue is introduced with a pipette or platinum loop under aseptic conditions into the condensation liquid at the bottom of the tube. It is well to prevent evaporation either by capping the tubes with rubber caps or sheets of tin-foil, or by dipping the cotton plug into boiling paraffin before inserting it into the culture tube.

This method has been modified for various purposes (see Nöller, 1917, and Hoare, 1923).

The *Leptospira* medium devised by Noguchi (1924) has been used for the cultivation of various species of TRYPANOSOMIDÆ (see Chapter XXVIII for details).

For culturing the pathogenic trypanosomes Ponselle (1923), has found the following satisfactory:

Sodium chloride	0.3 to 0.8 gm.[16]
Peptone (Witte's)	2.0 gms.
Gelatin	2.0 gms.
Normal solution of sodium carbonate	1.0 c.c.
Distilled water	100.0 c.c.

Ingredients must be chemically pure. They are dissolved by heat and the solution is autoclaved. When it has cooled to laboratory temperature, it is enriched by the addition of an equal volume of sterile rabbit's serum for primary cultures, or of sterile defibrinated blood for subculturing. The mixture is tubed in three cubic centimeter quantities and is inactivated by heating at 56° C. for thirty minutes. It is then incubated to test for sterility.

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[16] Varies according to species of trypanosome: 0.3 gm. for *T. brucei,* 0.6 gm. for *T. pecaudi,* and 0.8 gm. for *T. rhodesiense* and *T. dimorphon.*

Malarial parasites and piroplasms. The methods used for the cultivation of these two groups of organisms is the same. The procedure consists in growing the parasites in a biological fluid—serum, ascitic or hydrocele fluid—plus blood cells and a small amount of dextrose. The earlier investigators believed that anaërobic conditions were essential and that the removal of leucocytes by centrifugation greatly reduced the mortality of the extracellular forms, but subsequent work tends to indicate that neither of these precautions is necessary. The method adapted from the work of Bass and Johns (1911, 1912, 1914), is as follows:

Primary culture. Blood containing recently segmented or, in the case of the piroplasms, budded forms is drawn into a container in which there is a sufficient quantity of a fifty per cent solution of dextrose so that there will be 0.1 of a cubic centimeter of the solution to each ten cubic centimeters of blood. The mixture is defibrinated by stirring and is distributed under aseptic conditions into culture tubes in from five to eight cubic centimeter amounts. The tubes are incubated in the vertical position at 38°-40° C. Cells from the upper layers of the column are examined for parasites.

Sub-cultures. It is not easy to carry cultures of these parasites through many asexual generations. Bass and Johns obtained growth following transfer of the primary culture into tubes containing the serum and erythrocytes of normal defibrinated blood plus dextrose. They obtained these components by centrifuging normal defibrinated dextrose-blood until the leucocytes were separated from the red blood cells and the serum into the "buffy coat." The serum was pipetted into culture tubes without disturbing the layer of leucocytes, and the red blood cells were removed by passing a pipette through the layer of white cells and drawing off the erythrocytes. The primary culture is centrifuged in the same manner, and infected red blood cells free from leucocytes are used to seed the sub-culture. Subcultures rarely last over three generations, and the highest recorded number of generations in vitro is five.

This method has been modified by various workers so as to use a smaller quantity of blood, to provide anaërobic conditions, etc. For these particulars see Thomson and Thomson (1913), Row (1917), Sinton (1922), and Ioff (1925).

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